focused on immune response induced by EP4 signaling and demonstrated in vitro that the EP4 antagonist ONO-AE3-208 enhanced, whereas the EP4 agonist ONO-AE1-734 suppressed, the proliferation of and Th1 cytokine production by lamina propria mononuclear cells from the colon (Kabashima et al., 2002). Similarly, Nitta et al. (2002) reported that the EP4 agonist ONO-AE1-329 suppressed rat dextran sodium sulfate–induced colitis through upregulation of the anti-inflammatory cytokine IL-10. Jiang et al. (2007) demonstrated that treatment with the EP4 agonists ONO-AE1-329 or AGN205203 ameliorated a murine model of colitis, and that EP4 activation decreased colon epithelial apoptosis, prevented goblet cell depletion, and promoted epithelial regeneration in vitro. These studies suggest that EP4 maintains gastrointestinal homeostasis by preserving mucosal integrity and downregulating inflammatory immune response.

e. Inflammatory bowel disease. In contrast to its protective effect on gastrointestinal mucosal integrity, more recent studies have suggested that the EP4 receptor plays proinflammatory roles in inflammatory bowel disease. Inflammatory bowel disease, including Crohn's disease and ulcerative colitis, is chronic and relapsing and is characterized by inflammation in the large and/or small intestine associated with diarrhea, occult blood, and abdominal pain. Studies in humans have implicated impaired mucosal barrier function, production of proinflammatory cytokines, and activation of CD4+ T cells in the pathogenesis of inflammatory bowel disease (Fiocchi, 1998). It is known that PGE_2 is produced abundantly and that EP4 is upregulated in the affected intestine (Hommes et al., 1996; Lejeune et al., 2010). Furthermore, in a genome-wide association study of Caucasian patients with Crohn's disease, Libioulle et al. (2007) identified a region of approximately 250 kb on chromosome 5p13.1. Studies in lymphoblastoid cell lines revealed that genetic variants in the Crohn's disease–associated region influence the expression levels of the closest known gene, PTGER4, located 270 kb away in the direction of the centromere. This study suggested that genetic variants associated with Crohn's disease on chromosome 5p13.1 could modulate *cis*-acting regulatory elements of PTGER4 (Libioulle et al., 2007). From these observations, EP4 is likely to regulate Crohn's disease initiation and progression.

Th17 cells have emerged recently as central players in various inflammatory/autoimmune conditions, including inflammatory bowel disease (Yen et al., 2006). Crohn's disease patients have increased levels of IL-17 in serum and intestinal mucosa (Fujino et al., 2003b; Nielsen et al., 2003; Fuss et al., 2006). In accordance with these data, Sheibanie et al. (2007b) demonstrated that the PGE analog misoprostol exacerbated 2,4,6-trinitrobenzene sulfonic acid–induced colitis and that this exacerbation was correlated with an increase in IL-23 and IL-17, a decrease in IL-12p35 expression in the colon and mesenteric lymph nodes, and a substantial increase in the numbers of infiltrating neutrophils and Th17 cells in the colonic tissue. The study demonstrated that PGE_2 promotes IL-23 and inhibits IL-12 and IL-27 expression through EP4 in dendritic cells in vitro, suggesting that PGE_2 exacerbates the inflammatory process through the release of dendritic cell–derived IL-23 and the subsequent support of the autoreactive/inflammatory Th17 phenotype (Sheibanie et al., 2007b). Taken together, these data suggest that increased expression and subsequent activation of EP4 contribute to exacerbation of Crohn's disease through enhancement of the Th17 immune response.

IL-8 is a potent neutrophil chemoattractant and activator, and its levels correspond to the active grade of inflammatory bowel disease, including ulcerative colitis and Crohn's disease (Mazzucchelli et al., 1994). Srivastava et al. (2012) examined the mechanisms of differential regulation of IL-8 production by EP4 and demonstrated that PGE_2-EP4 signaling activated CREB through both the PKA and PI3K pathways. Interestingly, they also demonstrated that EP2 activated the transcription factor-inducible cAMP early repressor (Srivastava et al., 2012). Because inducible cAMP early repressor lacks the transactivation domain, it functions as a transcription repressor, unlike CREB. These data suggest that PGE_2 coupling through EP4 and EP2 receptors can therefore act in an opposing manner to either promote (EP4) or decrease (EP2) IL-8 expression, even though both receptors use the same second messenger, cAMP. Since Chadee's laboratory has reported that PGE_2 promotes IL-8 production in the human colonic epithelial cell lines Caco-2 and T84 through both the PKA and PI3K pathways (Dey and Chadee, 2008; Dey et al., 2009; Srivastava et al., 2012), it appears that EP4 contributes to IL-8 production.

f. Epithelial barrier. More recent data have suggested that colonic epithelial barrier function is disrupted by EP4 signaling in Caco-2 and T84 cells (Lejeune et al., 2010, 2011; Rodriguez-Lagunas et al., 2010). It has been reported that EP4 signaling increased intracellular Ca^{2+} concentration through the cAMP-PKA pathway, resulting in disruption of the colonic epithelial barrier in vitro (Rodriguez-Lagunas et al., 2010). Recently, several authors have described the cross-talk relationship between IP_3 receptors/Ca^{2+} release and the cAMP-PKA signaling pathway. Phosphorylation of IP_3 receptors by PKA results in a significant enhancement of IP_3-induced intracellular Ca^{2+} and is involved in diverse Ca^{2+}-regulated physiologic processes (Bruce et al., 2002; Tang et al., 2003; Chaloux et al., 2007). Wagner et al. (2008) demonstrated that PKA phosphorylation increases the sensitivity of the IP_3 receptor to IP_3. Intestinal epithelial barrier function may be regulated by these mechanisms.

Collectively, the evidence indicates that EP4 signaling maintains gastrointestinal homeostasis by preserving mucosal function in physiologic settings. However, in chronic inflammatory bowel disease such as Crohn's disease, EP4 signaling is suggested to promote intestinal inflammation.

F. Renal System

1. Expression. Northern blot analysis demonstrated moderate but significant expression of the EP4 receptor in the kidneys of humans (An et al., 1993; Bastien et al., 1994), mice (Sugimoto and Narumiya, 2007), rats (Sando et al., 1994), and rabbits (Breyer et al., 1996b). In situ hybridization studies have revealed that the EP4 receptor is highly expressed in the glomerulus (Sugimoto et al., 1994). The EP3 receptor, on the other hand, is expressed in the tubular epithelium, the thick ascending limb, and the cortical collecting ducts in the outer medulla. The EP1 receptor is expressed in the papillary collecting ducts. Similarly, EP4 receptor mRNA is predominantly expressed in the glomerulus in humans (Breyer et al., 1996b) and rabbits (Breyer et al., 1996a; Morath et al., 1999), suggesting that EP4 contributes to the regulation of glomerular hemodynamics and renin release (Breyer and Breyer, 2001). In the normal and ischemic adult human kidney, vascular COX-2 was colocalized with EP4 receptors (Therland et al., 2004). In a study in rats, the EP4 receptor was strongly expressed in the glomeruli, renin-secreting juxta-glomerular granular (JG) granular cells (Jensen et al., 1999), glomerular epithelial cells (Aoudjit et al., 2006), distal convoluted tubules, cortical collecting ducts (Jensen et al., 2001), and developing renal tubules (Yamamoto et al., 2011). When rats were given low-NaCl diets, the EP4 transcripts in glomeruli were significantly increased, implicating its role in regulating NaCl homeostasis (Jensen et al., 1999).

2. Function. The dominant expression of EP4 in the glomerulus suggests that EP4 may regulate glomerular filtration. Albuminuria is a useful marker for evaluation of glomerular filtration barrier (GFB) damage. Conventionally, nonsteroidal anti-inflammatory drugs have been reported to reduce proteinuria (Vriesendorp et al., 1986), suggesting that prostanoids, derived from COX-1 or COX-2, may worsen GFB damage. The effect of EP4 receptor signaling on glomerular function has remained controversial, however.

Animal studies using podocyte-specific EP4 receptor-overexpressing or EP4 receptor–deficient mice were performed by Stitt-Cavanagh et al. (2010). They induced renal ablation by 5/6 nephrectomy, and found increased proteinuria and mortality in mice overexpressing the EP4 receptor (Stitt-Cavanagh et al., 2010). In EP4-deficient mice, however, proteinuria was decreased and glomerular lesions became milder. A COX-2 inhibitor also decreased proteinuria. They also found that EP4 receptor overexpression in cultured podocytes resulted in enhanced susceptibility to mechanical stretch-induced detachment from culture dishes, which may be a potential mechanism leading to the pathogenesis of proteinuria. Thus, PGE_2, acting via EP4 receptors, may progress podocyte injury and GFB damage, leading to proteinuria.

In contrast to the previous findings, Aoudjit et al. (2006) reported that an EP4 receptor antagonist worsened proteinuria and glomerular apoptosis in a rat model of podocyte injury. Similarly, Nagamatsu et al. (2006) demonstrated that an EP4 receptor agonist was protective in antiglomerulus antiserum–induced glomerulonephritis in mice. It was most likely that EP4/cAMP signaling enhanced clearance of aggregated protein from the glomeruli (Nagamatsu et al., 2006). Thus, it seems that the EP4 receptor is, at the least, involved in the development of glomerular diseases, but further studies are required to reveal its mechanistic role.

Meanwhile, EP4 may regulate renal circulation, and thus may contribute to glomerular injury. PGE_2 mediates vasodilatory effects in the preglomerular circulation; this is the mechanism by which NSAIDs reduce glomerular filtration rate and renal blood flow (Schnermann and Weber, 1982; Chaudhari et al., 1990). Edwards (1985) reported that PGE_2 exerted a vasorelaxing effect on the afferent arteriole, but not on the efferent arteriole of rabbit glomeruli.

EP4 may play a role in renin secretion. PGE_2-induced renin release through EP4 receptors in mice was demonstrated in the isolated perfused kidney (Schweda et al., 2004) and in isolated JG cells (Friis et al., 2005). These studies found that EP4 stimulation caused PKA-mediated exocytotic fusion and release of renin granules in rat JG cells. In accordance with these findings, plasma renin concentrations were significantly lower in EP4 receptor–deficient mice than in wild-type mice. Moreover, a low dose of PGE_2 failed to induce renin secretion in the isolated kidneys of EP4 receptor–deficient mice, whereas the same dose of PGE_2 enhanced renin secretion in wild-type and other EP receptor–deficient kidneys. These findings indicate that the EP4 receptor may play a critical role in the regulation of renin secretion under normal conditions. Cyclic AMP has been recognized as an important regulator of renin secretion (Hackenthal et al., 1990). Aldehni et al. (2011) reported that AC5 and AC6, which are $G_i\alpha$- and Ca^{2+}-inhibitable AC isoforms, are involved in the stimulatory effect of catecholamines and that PGE_2-mediated signaling is involved the secretion of renin. Isoproterenol- and PGE_2-induced renin secretion was attenuated in isolated perfused kidneys from AC5- and AC6-deficient mice. Furthermore, EP2 stimulation caused PKA-mediated release of renin granules in rat JG cells (Friis et al., 2005). These studies suggest that renin secretion might be related to cAMP downstream of EP4 signaling.

EP4 is also expressed in the distal convoluted tubule and the cortical collecting duct (Olesen et al., 2011). A recent report demonstrated that EP4 receptor agonists increase aquaporin-2 phosphorylation and trafficking. EP4 may be involved in the regulation of water homeostasis via the regulation of water transport in the collecting duct. cAMP has been demonstrated to play an important role in the regulation of water transport in the collecting duct. Vasopressin exerts its antidiuretic effect through vasopressin 2 receptors coupled to Gs protein, which activates AC to form cAMP from ATP. Increased cAMP activates PKA, which phosphorylates aquaporin-2 water channels, thereby promoting water reabsorption (Nielsen et al., 1999). Further studies are required to determine whether EP4 is involved in the regulation of water homeostasis in the collecting duct.

G. Reproductive System

1. Expression. PGE_2 regulates various uterine functions, such as contraction and relaxation of the uterine smooth muscles, cervical ripening and labor induction, elevation of endometrial vascular permeability, and induction of decidualization (Murdoch et al., 1993). In female reproductive organs such as the ovary and uterus, hormonal exposure induces expression of the EP subtypes in a cell type–specific manner. EP4 is expressed in the mouse ovary (Segi et al., 2003) and in the human (Milne et al., 2001; Astle et al., 2005), baboon (Smith et al., 1998), mouse (Katsuyama et al., 1997; Yang et al., 1997), rat (Blesson et al., 2012), and guinea pig (Terry et al., 2008) uterus, although Arosh et al. (2003, 2004) reported that EP4 mRNA was undetectable in the bovine uterus.

In the mouse ovary, EP4 expression was found in oocytes in the preantral follicles. Upon gonadotropin stimulation, however, it disappeared, reappearing in both cumulus and granulose cells 3 hours after gonadotropin stimulation. EP4 mRNA was detected in the epithelium throughout the oviduct, the tube extending from the periovarial space to the uterine horns (Segi et al., 2003).

Milne et al. (2001) examined menstrual cyclical variation in endometrial human EP receptor mRNA expression. They demonstrated that EP4 receptor expression was significantly higher in the late proliferative stage than in the early, middle, and late secretory stages. Both EP4 and EP2 receptor expression were found in endometrial glandular epithelial and vascular cells, with no notable spatial or temporal variation (Milne et al., 2001). The expression change in pseudopregnancy was also demonstrated in the mouse uterus. The EP4 receptor transcripts were expressed mainly in the luminal epithelium during peri-implantation; they were increased in endometrial stromal cells and the glandular epithelium after pharmacological induction of pseudopregnancy (Katsuyama et al., 1997; Yang et al., 1997). EP4 transcripts were also present in the myometrium and remained unchanged throughout gestation in pregnant humans (Astle et al., 2005) and guinea pigs (Terry et al., 2008). EP3 receptor mRNA was predominantly expressed in the myometrium (Katsuyama et al., 1997; Yang et al., 1997).

The uterine cervix also plays a crucial role in pregnancy; it must remain closed during gestation, then soften and dilate during labor. PGE_2 has been used to induce cervical ripening for many years (Woodward and Chen, 2004). EP4 receptor expression has been found in smooth muscle cells and epithelial cells in the cervix, and at its highest concentration at parturition in goats (Gu et al., 2012) and rats (Chien and Macgregor, 2003; Hinton et al., 2010), suggesting that increased EP4 receptor expression may regulate cervical relaxation. EP4 receptor expression is also increased in cervical inflammation. *Chlamydia trachomatis* LGV2 selectively upregulated COX-2 and EP4 in cervical epithelial HeLa 229 cells (Fukuda et al., 2005). Similarly, EP4 receptor was increased in LPS-treated rabbit interstitial cells in the cervix (Fukuda et al., 2007) and in IL-1β–treated human cervical fibroblasts (Schmitz et al., 2003).

EP4 receptor may be involved in the male reproductive organs as well. Moderate expression of EP4 was demonstrated in the human (An et al., 1993), bovine (Arosh et al., 2003), and chicken (Kwok et al., 2008) testis, although no EP4 expression was detectable in the mouse (Honda et al., 1993) or rat (Sando et al., 1994) testis.

2. Function. Myometrial relaxation is mediated by EP4 as well as by EP2 receptors (Senior et al., 1993; Negishi et al., 1995). The localization and expression of these receptors are thus involved in the onset and maintenance of labor. However, no significant pregnancy- or labor-associated changes in EP4 receptor expression were reported in the human uterus (Astle et al., 2005). The EP4 receptor is similarly expressed in the upper and lower segments of the uterus. It is most likely that EP4 does not play a major role in PGE_2-mediated regulation of myometrial tone during pregnancy and labor. Cyclic AMP may play an important role in myometrial quiescence (Yuan and Lopez Bernal, 2007). In general, PKA phosphorylates cellular proteins that may cause smooth muscle relaxation, including myosin light-chain kinase (Nishikawa et al., 1984), PDE4 (Murthy et al., 2002), and PLC. EP4 signaling may be involved in the enhancement of such phosphorylation, and it remains as yet a possibility that EP4 is involved in other relevant processes during pregnancy and delivery. Glycosaminoglycan redistribution is an important process involved in cervical ripening; Schmitz et al. (2001) demonstrated that, of the four subtypes of PGE_2 receptors, only EP4 mediated PGE_2-induced glycosaminoglycan synthesis in human cervical fibroblasts in a PKA-independent manner.

The EP4 receptor may regulate endometrial function. PGE_2 promotes the survival of human endometriotic cells through the EP4 receptors by activating ERK, Akt, NF-κB, and the β-catenin signaling pathway (Banu et al., 2009). Inhibition of EP4 may suppress proliferation and induce apoptosis of human endometriotic cells. In addition, Lee et al. (2012) found that EP4 was expressed in the ovine endometrium, especially during pregnancy. Interferon-τ, a pregnancy recognition signal in ruminants, increased EP4 receptors in the endometrium.

H. Lungs

1. Expression. The lung is an organ in which the EP4 receptor is abundantly expressed in many species, including humans, mice, rats, and rabbits (An et al., 1993; Honda et al., 1993; Bastien et al., 1994; Sando et al., 1994; Breyer et al., 1996b). Anatomically, the lung is composed of the bronchial tree, the alveoli, and a dense vascular network, including a variety of cell types. EP4 is highly expressed in airway smooth muscle cells, pulmonary fibroblasts, and smooth muscle cells of the pulmonary vein. In particular, together with the EP2 receptor, EP4 transcripts and proteins are abundantly expressed in human airway smooth muscle cells (Bradbury et al., 2005; Clarke et al., 2005; Mori et al., 2011; Benyahia et al., 2012). It is also known that EP4 activation causes potent relaxation in human and rat bronchial preparations (Lydford and McKechnie, 1994; Benyahia et al., 2012).

Pulmonary fibroblasts are important in the development and maintenance of lung structure and function. Their proliferation and phenotypic changes play critical roles in normal tissue repair as well as the development of pulmonary fibrosis (Ramos et al., 2001). The EP4 receptor is expressed in both fetal (Choung et al., 1998; Li et al., 2011) and adult lung fibroblasts (Huang et al., 2007; Nikam et al., 2011) in humans. Togo et al. (2008) demonstrated that the EP2 and EP4 receptors were expressed in normal pulmonary fibroblasts and that these receptors were increased in fibroblasts from patients with chronic obstructive pulmonary disease where they contribute to the pathogenesis of emphysema.

In the human pulmonary vasculature, the EP4 receptor is mostly expressed in the smooth muscle layer of the vein and only weakly in the artery (Walch et al., 1999; Foudi et al., 2008), suggesting that EP4 induces relaxation of the vein. This expression pattern may change under disease conditions. Lai et al. (2008) reported that EP4 expression in the artery is readily detectable in pulmonary arterial hypertension in human and rat models. In other cell types in the lung, the EP4 receptor is found in human pulmonary microvascular endothelial cells (Aso et al., 2012), the human bronchial epithelial cell line BEAS-2B (N'Guessan et al., 2007), and human alveolar macrophages (Ratcliffe et al., 2007).

2. Function. EP4 may induce relaxation of the airway and inhibit smooth muscle cell proliferation. Buckley et al. (2011) reported that PGE_2-induced relaxation of the airway was mediated through EP4 in humans and rats. Mori et al. (2011) demonstrated that PGE_2 inhibited fetal bovine serum–induced proliferation of human airway smooth muscle cells via EP4 receptor activation. Taken together, these findings suggest that the EP4 receptor could potentially be a therapeutic target in treating pulmonary diseases such as asthma and chronic obstructive pulmonary disease. As they potentially occur downstream of EP4/cAMP signaling, both PKA and Epac are involved in anti-inflammatory (Oldenburger et al., 2012) and relaxation (Zieba et al., 2011) signaling in airway smooth muscle cells. It was also demonstrated that PGE_2 inhibits Platelet-derived growth factor-induced phenotype switching of tracheal smooth muscle cells, from a contractile to a proliferative phenotype, through the activation of the cAMP effectors PKA and Epac (Roscioni et al., 2011).

In fibroblasts, PGE_2 inhibits proliferation and collagen synthesis in human lungs (Huang et al., 2008). Proliferation and collagen synthesis were likewise attenuated by the activation of the cAMP effectors PKA and Epac, respectively. The accumulation of cAMP was promoted by EP4 receptor activation. In addition, chemotaxis of human lung fibroblasts was inhibited by EP4 (Li et al., 2011). Subtype-specific modulation of EP receptor activity could potentially be a new therapy for fibrotic lung disease.

An interesting vasodilatory effect of EP4-mediated signaling has been reported. Cyclic AMP accumulation in vascular smooth muscle cells is thought to be the main mechanism of prostanoid-induced vasorelaxation. Lai et al. (2008) demonstrated that iloprost, a stable analog of PGI_2, increased cAMP via the EP4 receptor in pulmonary arterial smooth muscle cells isolated from rats with pulmonary hypertension. Similarly, Foudi et al. (2008) reported that PGE_2-induced vasorelaxation of the human pulmonary vein was also mediated by the EP4 receptor. It has been demonstrated that IP receptors are downregulated in human pulmonary artery hypertension, whereas the EP4 receptor is stably expressed. The EP4 receptor could thus be a novel effective therapeutic target for the treatment of pulmonary artery hypertension.

I. Skin

1. Expression. Tober et al. (2007) reported that the EP4 receptor was abundant in epidermal keratinocytes, dermal leukocytes, and vascular endothelium in murine skin. UV-B exposure induced EP4 relocalization to the plasma membranes of keratinocytes, whereas its diffuse cytoplasmic staining pattern was unchanged in the rest of the epidermis. EP4 expression was also detected in sebocytes (Chen et al., 2009), hair follicles (Colombe et al., 2008), melanoma cells (Singh and Katiyar, 2011), and squamous cell carcinoma (Lee et al., 2005) in humans. Kabashima et al. (2003) reported the expression of EP4 receptor transcripts in Langerhans cells prepared from epidermis. Li et al. (2000) reported that EP4 receptor mRNA was upregulated in fetal rabbit skin wounds, yet downregulated in adult rabbit skin wounds.

2. Function. EP4 is involved in skin inflammation. It has been suggested that PGE_2 is upregulated within antigen-exposed skin (Ruzicka and Printz, 1982; Eberhard et al., 2002). Kabashima et al. (2003) demonstrated that PGE_2 promoted skin immune responses by enhancing the migration and maturation of Langerhans cells through EP4 signaling. Although the transcripts of all four PGE_2 receptor subtypes were detected in Langerhans cells, only EP4 deletion inhibited Langerhans cell accumulation in regional lymph nodes after application of fluorescein isothiocyanate to the skin. In addition, the immune responses in a dinitrofluorobenzene-induced contact hypersensitivity model were significantly attenuated in EP4 receptor KO mice and in EP4 antagonist-treated wild-type mice (Kabashima et al., 2007). Chun et al. (2007) suggested that PGE_2 exerted an antiapoptotic effect in UV-B–exposed mouse skin through EP4/PKA/Akt signaling. Thus, EP4 is suggested to promote immune response in skin, although the downstream signaling process of EP4 remains largely unknown.

J. Nervous System

1. Expression. Zhang and Rivest (1999) reported the distribution of EP4 receptor transcripts in the rat brain. The localization of the EP4 receptor was distinct from that of the EP2 receptor. EP4 receptors were mainly expressed in regions involved in the regulation of neuroendocrine and autonomic activities. Southall and Vasko (2001) demonstrated EP4 receptor expression in embryonic rat sensory neurons and adult rat dorsal root ganglia cells.

2. Function. Traditional NSAIDs exert their antinociceptive effects through the inhibition of prostaglandin. Accordingly, prostaglandin-mediated signaling has been thought to be involved in the development of inflammatory pain. Thermal and mechanical hyperalgesia, mechanical allodynia, and joint pain were suppressed by EP4 antagonists (Lin et al., 2006; Kassuya et al., 2007; Nakao et al., 2007; Clark et al., 2008; Murase et al., 2008). Southalland Vasko (2001) demonstrated that EP4 receptors mediated the PGE_2-induced sensitization of sensory neurons. PGE_2-induced accumulation of cAMP and release of immunoreactive substance P and calcitonin gene-related peptide, all of which play important roles in the development of pain and hyperalgesia, were blocked by downregulation of EP4 receptors. Because the cAMP-PKA pathway is involved in the development of hyperalgesia after injury in the dorsal root ganglion (Song et al., 2006), EP4 may activate this pathway to regulate hyperalgesia.

Fever production may involve EP4 signaling. Oka et al. (2000) demonstrated that the EP4 receptor was expressed in regions that are involved in PGE_2-induced fever responses, including the organum vasculosum of the lamina terminalis and the adjacent preoptic area. Several reports have indicated that the EP4 receptor may contribute to PGE_2-induced changes in body temperature (Oka et al., 2000, 2003).

EP4 may also play a role in neuronal degeneration and regeneration. Hoshino et al. (2007) reported that PGE_2 enhanced the production of amyloid-β through the EP4 receptors in human neuroblastoma cells. Moreover, they observed that cognitive function of mice in an Alzheimer's disease model was improved by genetic and pharmacological inhibition of EP4 (Hoshino et al., 2012). In contrast, Liang et al. (2011) reported that an EP4 receptor agonist exerted a protective effect against cerebral ischemia injury in mice. Deletion of neuronal EP4 increased the severity of cerebral injury, as did endothelial deletion of EP4. The effect of EP4 on cerebral perfusion via endothelial nitric-oxide synthase function may be involved in such beneficial roles. A neuroprotective effect of EP4 signaling has been reported in various other models, e.g., a mouse multiple sclerosis model (Esaki et al., 2010), a rat spinal cord injury model (Umemura et al., 2010), and a mouse *N*-methyl-D-aspartate–mediated acute brain damage model (Ahmad et al., 2005). Taken together, these data suggest that EP4 contributes to hyperalgesia and fever production and plays protective roles in neuronal degeneration and regeneration, although its precise downstream signaling pathways have not been reported.

K. Other Systems

Prostanoids play a critical role in the regulation of platelet function. Several reports have indicated that EP4 mediates antithrombotic signaling (Iyu et al., 2010; Kuriyama et al., 2010; Philipose et al., 2010). Philipose et al. (2010) have reported that an EP4 agonist inhibited platelet aggregation, adhesion of platelets to fibrinogen, and thrombus formation in vitro. EP4 receptor activation could thus be a novel target for antithrombotic therapy. EP4 is also expressed in the cochleae (Hori et al., 2009). Hori et al. (2009) reported that local EP4 agonist treatment improved noise-induced hearing loss in guinea pigs. Finally, Woodward et al. (2009) reported that an EP4 receptor agonist potently decreased intraocular pressure in laser-induced ocular hypertensive monkeys. EP4 could potentially be a new therapeutic target for antiglaucoma therapy.

IV. Conclusions

This article presents an overview of the functions of EP4 and its intracellular signaling pathways in physiologic and pathologic conditions. EP4 was originally identified as a G_s-coupled receptor and has been recognized to produce cAMP. Recent emerging evidence has revealed that, in addition to cAMP and its downstream signaling, EP4 also modulates a variety of signaling pathways, such as PI3K, β-arrestin, and transactivation of EGFR. The roles of these EP4-mediated pathways in physiologic and pathologic processes continue to be discovered.

Among the EP receptors, EP4 is reported to be most abundantly expressed in the heart, the ductus arteriosus, monocytes/macrophages, bone, and the colon. It maintains the physiologic functions of these organs through protein synthesis, a vasodilatory effect, regulation of immune response, anabolic effect, and mucosal barrier function, respectively. EP4 is also highly expressed in pathologic conditions, such as colorectal cancer, inflammatory bowel disease, rheumatoid arthritis, atherosclerotic plaque, and aortic aneurysm. Studies using mouse lines devoid of each of the four EP receptors further support the concept that EP4, but not the other EP receptors, plays a primary role in bone metabolism, osteoarthritis, and immune response in the skin. Therefore, the EP4 receptor appeared to be an attractive target by which to affect manifestations of various pathologic states by application of either agonists or antagonists of the receptor. In particular, EP4 agonists have drawn much attention for their promotion of osteogenesis and their suppression of colitis, and the potential usefulness of an EP4 agonist as a treatment of bone diseases or inflammatory bowel disease has been examined in clinical trials. EP4 antagonists may be suitable for use in the treatment of rheumatoid arthritis and osteoarthritis, where continuous dosing demands a drug with a superior safety profile. Traditional NSAIDs and COX inhibitors affect a number of other related prostaglandins and can cause serious side effects. The potential of an EP4 antagonist to improve prognosis in colon cancer, myocardial infarction, aortic aneurysm, neovascularization, and autoimmune encephalomyelitis is also of great interest.

Interestingly, modulating EP4 signaling could work on more than one mechanism, because EP4 is distributed in various organs and circulating immune cells. For instance, inhibition of EP4 signaling has been expected to be useful as a treatment of migraine due to its cerebral vasoconstrictive and immunosuppressive effects. The possibilities for such dual mechanisms of action of EP4 signaling in pathologic conditions of various organs should be explored. In particular, it will be important to further clarify the intracellular signaling pathways and the precise molecular mechanisms involved in EP4-mediated pathophysiologic actions. These additional studies should lead to significant opportunities for new pharmacological therapies.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Yokoyama, Iwatsubo, Umemura, Fujita, Ishikawa.

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Type 5 Adenylyl Cyclase Increases Oxidative Stress by Transcriptional Regulation of MnSOD via the SIRT1/FoxO3a Pathway

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Abstract

Background—For reasons that remain unclear, whether type 5 AC (AC5), one of two major AC isoforms in heart, is protective or deleterious in response to cardiac stress is controversial. To reconcile this controversy we examined the cardiomyopathy induced by chronic isoproterenol (ISO) in AC5 transgenic (Tg) mice and the signaling mechanisms involved.

Methods and Results—Chronic ISO increased oxidative stress and induced more severe cardiomyopathy in AC5 Tg, as left ventricular (LV) ejection fraction fell 1.9 fold more than wild type (WT), along with greater LV dilation and increased fibrosis, apoptosis and hypertrophy. Oxidative stress induced by chronic ISO, detected by 8-OhDG was 15% greater, $p=0.007$, in AC5 Tg hearts, while protein expression of MnSOD was reduced by 38%, indicating that the susceptibility of AC5 Tg to cardiomyopathy may be due to decreased MnSOD expression. Consistent with this, susceptibility of the AC5 Tg to cardiomyopathy was suppressed by overexpression of MnSOD, whereas protection afforded by the AC5 KO was lost in AC5 KO×MnSOD$^{+/-}$ mice. Elevation of MnSOD was eliminated by both sirtuin and MEK inhibitors, suggesting both the SIRT1/FoxO3a and MEK/ERK pathway are involved in MnSOD regulation by AC5.

Conclusion—Overexpression of AC5 exacerbates the cardiomyopathy induced by chronic catecholamine stress by altering regulation of SIRT1/FoxO3a, MEK/ERK and MnSOD, resulting in oxidative stress intolerance, thereby shedding light on new approaches for treatment of heart failure.

Keywords

Adenylyl cyclase; Adrenergic; Cardiomyopathy; Oxidative Stress

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Disclosures

Dr. David A. Sinclair is a consultant to Sirtris, a GSK company working to develop sirtuin-targeted medicines.

Introduction

Adenylyl cyclase (AC) is a key regulator of health and longevity in organisms ranging from yeast to mammals.[1–5] In the heart AC is a critical link in sympathetic control and beta adrenergic receptor (beta-AR) signaling and therefore plays a fundamental role in mediating not only baseline cardiac function, but also the cardiac response to stress, e.g., in the pathogenesis of heart failure. Type 5 AC (AC5) is one of two major isoforms in heart, the other being type 6 AC (AC6). For reasons that remain unclear, whether AC5 is protective or deleterious in response to cardiac stress is controversial, particularly with respect to the signaling mechanisms involved, and whether these mechanisms are shared by AC6. It is generally accepted that cardiac-specific AC5 overexpressed (AC5 Tg) mice exhibit enhanced cardiac performance,[6] which follows from the role of AC, which generates cyclic AMP upon beta-AR stimulation resulting in increased cardiac contractility and heart rate. However, the extent to which altered AC5 regulation is protective with chronic stress remains controversial. Prior studies examined whether overexpression or disruption of AC5 in the heart could affect the progression of cardiomyopathy induced by overexpression of Galphaq and beta1-AR. This was accomplished by mating overexpressed Galphaq and beta1-AR with AC5 Tg or AC5 knockout (KO) mice. These studies found that AC5 Tg rescued Galphaq cardiomyopathy,[6] but not beta1-AR cardiomyopathy,[7] and AC5 KO mice failed to rescue cardiomyopathy in Galphaq mice.[8] In addition, AC5 KO mice rescued cardiomyopathies from chronic pressure overload,[9] chronic catecholamine stress,[10] and aging.[1]

Since beta-AR signaling, of which AC is central, plays a key role in the pathogenesis of heart failure and since beta-AR blockade therapy is widely used in patients with heart failure, but that therapy is still far from perfect, it becomes critical to reconcile the controversy and understand the role of AC in the heart in the development of cardiomyopathy and heart failure, which would eventually be of clinical importance. Accordingly, this was the overall goal of the current investigation. We first examined the extent to which manganese superoxide dismutase (MnSOD) regulation and oxidative stress were altered in AC5 Tg at baseline and in response to chronic beta-AR stimulation, since it is known that beta-AR stimulation increases oxidative stress,[11, 12] and that MnSOD is upregulated in AC5 KO mice.[1] The results of the experiments with bigenic mice (AC5 Tg × MnSOD Tg and AC5 KO × MnSOD$^{+/-}$) led us to elucidate the signaling mechanisms linking AC5, MnSOD and oxidative stress, and the involvement of the SIRT1/FoxO3a pathway. The SIRT1/FoxO3a pathway was selected to investigate, because MnSOD is upregulated in the AC5 KO mouse, which lives longer than wild type (WT)[1] and FoxO3a is the transcriptional factor most closely related to the anti-oxidative protective effects associated with longevity, as shown in several models: *C.elegans*,[13, 14] rats[15] and human quiescent cells.[16] The final goal was to investigate whether this pathway is regulated specifically by AC5, or whether it is common to all AC signaling in the heart, which would mean that these mechanisms were shared by the other major cardiac AC isoform, AC6.

Methods

Mouse Models

Generation of AC5 Tg mice was described previously.[17] AC5 KO × MnSOD$^{+/-}$ mice were generated by crossing AC5 KO mice with MnSOD heterozygous mice. AC5 Tg × MnSOD Tg were generated by crossing AC5 Tg mice with MnSOD Tg mice (From Jackson Laboratory, Stock ID: 009438). To produce catecholamine cardiomyopathy, ISO was delivered to 3–5 month old Tg mice, bigenic mice and corresponding control littermates for 7 days at a dose of 60 mg/kg/day with a miniosmotic pump (ALZET model 2001, DURECT Corp, Cupertino, California) as described.[10] The severity of the cardiomyopathy was assessed by echocardiographic measurements of LV ejection fraction and LV end diastolic and end systolic diameter and histopatholical measurements of myocardial fibrosis, apoptosis and myocyte cross sectional area. For the Tempol treatment group, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (Tempol, Sigma) was administered to AC5 Tg mice by dissolving it in drinking water at a concentration of 1mmol/L for 1 month prior to chronic ISO infusion to block oxidative stress. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, Eighth Edition 2011). This study was approved by the Animal Care and Use Committee at New Jersey Medical School.

Experimental procedures

All techniques are described in more detail in Supplemental materials with references to previous work with these techniques. Experimental procedures included: adenoviral construction (Figure S2), physiological studies,[10] primary culture of neonatal rat ventricular myocytes,[18] AC assay,[10] immunoprecipitation, western blotting,[1] quantitative RT-PCR,[18] 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA assay, chemiluminescent assay for superoxide production,[19] subcellular fractionation, luciferase activity, Chromatin Immunoprecipitation (ChIP) assay[15] and histological analyses (apoptosis, fibrosis and cell size).[20]

Statistical analysis

Normally distributed data were presented as mean±SEM. Otherwise, data were summarized using the Median and range. When the data were normally distributed, we used Student's unpaired t-test to compare two independent groups; otherwise, the difference was tested using the Mann-Whitney U test. For a comparison of three or more groups, one-way ANOVA was used if the sample population was normally distributed and within-group variances were approximately equal. The Student-Newman-Keuls test was used for post-hoc analysis. For data that did not meet the ANOVA assumptions, the Kruskal-Wallis test was applied and post hoc testing was carried out using the Mann-Whitney U test with Bonferroni correction. The Bonferroni correction factor is 3 for Figures 1, 5F and 5G. GraphPad-Prism 5.0 (GraphPad-Software, San Diego,CA), SPSS 20.0 (SPSS Inc, Chicago, IL) and SAS 9.3 (SAS, Research Triangle, NC) were used to perform the statistical analyses. P-values less than 0.05 defined statistical significance.

Results

AC5 Tg Mouse Model and Cardiomyopathy Induced by Chronic Isoproterenol (ISO)

AC5 protein expression, assessed by western blot analysis, was increased 26-fold in AC5 Tg (Figure S1A). Basal AC activity was increased 13-fold in AC5 Tg mice hearts compared to WT, and was increased 10-fold with forskolin compared to WT (Figure S1B). The AC5 Tg exhibited increased left ventricular ejection fraction (LVEF), p=0.0009, without ISO (WT=73(67–74)%; AC5 Tg=78(75–81)%) and heart rate was not significantly different, p=0.3176, (WT=337(325–465) bpm; AC5 Tg= 442(355–500)bpm). The increase in LVEF in response to an ISO challenge was similar in AC5 Tg and WT mice(Figure S1C).

Chronic ISO infusion induced more severe cardiomyopathy in AC5 Tg compared with WT, i.e., LVEF was lower, p=0.0058, in AC5 Tg (45(30–49)%) compared to WT (54(47–58)%). Actually the decline in LVEF was even more significant, since that takes into account the different baseline levels where LVEF was higher in AC5 Tg and fell to a lower level, p=0.0021 (Figure 1A). In addition, the LV dilated more in AC5 Tg mice than WT (Table S1). Similarly, chronic ISO induced more fibrosis (2.0-fold) and more myocyte apoptosis (2.8-fold) in AC5 Tg mice compared with WT (Figure 1B and 1C). There was also more LV hypertrophy, as measured by myocyte cross sectional area, but the increase (1.2-fold) was not as great as with fibrosis and apoptosis.

Overexpression of AC5 Increased Oxidative Stress

After chronic ISO stimulation, AC5 Tg mice exhibited 19% more GSSG content, an indicator of oxidative stress, than WT littermates (Figure 2A). Consistent with this, AC5 Tg mice had 15% more oxidative stress-induced DNA damage compared with WT mice after chronic ISO stimulation detected by 8-OHdG ELISA (Figure 2B). In AC5 overexpressed neonatal myocytes, superoxide production was approximately 2-fold more than in the control group (Figure 2C). AC5 knockdown (KD) myocytes increased cell survival with H_2O_2 treatment (Figure 2D). MnSOD is part of a mechanism that might be responsible for the opposite response of AC5 overexpressed (OE) and AC5 KD towards oxidative stress, since MnSOD is up-regulated in AC5 KO mice.

AC5 Down-Regulates MnSOD

By western blotting, the protein expression of MnSOD was reduced 38% in AC5 Tg mice compared with WT (Figure 3A). On the cellular level, 26% less MnSOD was detected in AC5 OE myocytes and MnSOD protein was increased a 2-fold and mRNA a 3.6-fold in AC5 KD myocytes (Figure 3B, 3C and 3D). The data demonstrated that AC5 regulated the protein and mRNA expression level of MnSOD, which altered MnSOD function.

MnSOD Overexpression Ameliorated Chronic ISO Cardiomyopathy in AC5 Tg

We increased MnSOD in AC5 Tg using a bigenic (AC5 Tg × MnSOD Tg) mouse. The cardiac specific MnSOD Tg mice had a 20-fold increase in SOD activity in the heart.[21] Baseline LVEF was similar in AC5 Tg × MnSOD Tg mice (85(84–89)%) and AC5 Tg mice (78(75–81)%). After chronic ISO, the LVEF of bigenic mice decreased significantly less (Figure 1A, Table S1), p=0.0033, to 74(66–77)%, than in AC5 Tg (45(30–49)%) mice (Figure 1A, Table S1). The increases in LVEDD and LVESD were also no longer greater than observed in WT (Table S1), and the increases in fibrosis and apoptosis, observed in AC5 Tg on chronic ISO, were no longer observed in the bigenic mice (Figure 1B and 1C). Similarly, Tempol, which also protects against oxidative stress, rescued the adverse effects of the AC5 Tg after chronic ISO stimulation, i.e., the LVEF in AC5 Tg with ISO and Tempol (63(43–69)%) was higher than with ISO in AC5 Tg without Tempol (45(30–49)%). Thus, these data demonstrated that down-regulation of MnSOD in AC5 Tg mice is a key mechanism mediating the exacerbated cardiomyopathy induced by chronic ISO.

Down-regulation of MnSOD Eliminated Protective Effects of AC5 KO under Chronic Catecholamine Stress

To investigate whether MnSOD was important for the protective effects of AC5 KO mice, we crossed the AC5 KO mice with MnSOD heterozygous KO mice. Previously, we reported that the AC5 KO mice were protected against catecholamine stress.[10] This was confirmed in the present study in a small cohort, where the fall in LVEF was less in the AC5 KO than WT with chronic ISO. This protection was lost in the bigenic mice, where LVEF after chronic ISO was decreased to 50(43–60)% (n=6), which was almost identical to the LVEF in the WT mice (53(39–62)%, n=11) (Table S1). Fibrosis, an indicator of the cardiomyopathy with chronic ISO was increased similarly in WT (2.69(1.64–3.84)%) and AC5 KO × $MnSOD^{+/-}$ mice (2.92(2.32–3.85)%) compared with AC5 KO mice with chronic ISO (1.13(0.81–1.37)%).