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Untersuchungen zur Regulation und Funktion der Cyclooxygenase-2 in Nierenmarkszellen unter osmotischem Stress

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1. Einleitung

Die Niere besitzt eine zentrale Rolle bei der Regulation des Elektrolyt-, Wasser- und Säure-Basen-Haushaltes. Komplexe, bedarfsorientierte Funktionsprozesse und Wechselwirkungen schaffen außerordentliche Lebensbedingungen für die Zellen des Organs. Durch das Gegenstromprinzip des Harnkonzentrierungsmechanismus entsteht ein cortico-papillärer osmotischer Gradient mit kontinuierlichem Anstieg der interstitiellen Teilchenkonzentration von der Nierenrinde zur Nierenpapille. Abhängig vom Hydratationsstatus des Organismus variieren die medullären Salz- und Harnstoffkonzentrationen erheblich. Die größten Osmolalitätsschwankungen finden sich entsprechend diesem Gradienten im Bereich der Papille (76). Bei ausgeglichenem Wasserhaushalt des Organismus finden sich dem Blutplasma nahezu isoosmolare Bedingungen von 300 mosmol/kg H_2O (3). In Antidiurese, dem Zustand der systemischen Dehydratation, werden in der Nierenpapille Osmolalitäten von bis zu 1200 mosm/kg H_2O erreicht. Zur Vermeidung einer „Auswaschung“ der zur effektiven Harnkonzentrierung benötigten hohen Salz- und Harnstoff-konzentrationen besteht im Nierenmark zudem ein relativ geringer Blutfluss, was konsekutiv einen niedrigen Sauerstoffpartialdruck bedingt (60). Die Zellen des Nierenmarkes besitzen spezifische adaptive und protektive Mechanismen um sich an derart lebensfeindliche Bedingungen anzupassen, in dieser Nierenregion zu überleben und ihre physiologische Funktion zu wahren.

1. 1. Osmoprotektive Gene

Die vor allem in Antidiurese vorherrschenden extrem hohen Teilchenkonzentrationen im Interstitium des Nierenmarkes bewirken die Expression sogenannter osmoprotektiver Gene. Zu diesen gehören unter anderem das Enzym Aldose-Reduktase (AR), der Betain-γ-Amino-Butyrat-Transporter 1 (BGT1), der Natrium-abhängige *Myo*-Inositol-Transporter (SMIT) sowie das Hitzeschockprotein 70 (HSP70) und die Cyclooxygenase-2 (COX-2).

Die hohen interstitiellen Osmolalitäten in der Medulla, die zur effektiven Harnkonzentrierung benötigt werden, induzieren ohne adaptive Mechanismen einen osmotisch bedingten Wasserausstrom aus den Zellen und führen hierdurch zu toxischen intrazellulären Ionenkonzentrationen (69). Daher akkumulieren in den Zellen des Nierenmarkes metabolisch neutrale, osmotisch wirksame Moleküle, sogenannte kompatible organische Osmolyte, welche ein entsprechendes osmotisches Gleichgewicht zum Extrazellulärraum herstellen. Von zentraler Bedeutung sind Sorbitol, Betain und *Myo*-Inositol (7, 54). Betain und *Myo*-Inositol akkumulieren in den Nierenmarkszellen durch gesteigerte Aufnahme über membranständige Transporter aus dem Extrazellulärraum. Betain wird über den Natrium- und Chlorid-abhängigen BGT1 in die Zellen aufgenommen (42). Die Anreicherung von *Myo*-Inositol wird durch den Natrium-abhängigen SMIT reguliert (62). Sorbitol akkumuliert durch vermehrte intrazelluläre Synthese durch das Enzym Aldose-Reduktase, welches die Reduktion von Glucose in Sorbitol katalysiert. Entsprechend dem cortico-medullären osmotischen Gradienten steigt die Expression von AR, SMIT und BGT1 vom Cortex bis zur Papille kontinuierlich an. Ihr quantitatives Vorkommen ist abhängig vom Diuresezustand des Organismus. In

Antidiurese, das heißt bei erhöhter interstitieller Osmolalität, wird die mRNA dieser Gene verstärkt exprimiert, während im Zustand der Diurese eine verminderte Transkription dieser Gene in der inneren Medulla nachweisbar ist (8).

Des Weiteren ist die Expression des HSP70 von protektiver Bedeutung. Das Chaperon bindet mit seiner Substrat-bindenden Domäne an hydophobe Peptidsequenzen von Proteinen (43). Die Expression von HSP70 wird bereits in der frühen Phase osmotischen Stresses induziert, es stabilisiert und schützt Substratproteine vor Denaturierung und Funktionsverlust (12, 53). Zusätzlich antagonisiert HSP70 Caspase-abhängige und Caspase-unabhängige Signalwege, die bei osmotischem Stress sowie bei erhöhten extrazellulären Harnstoff-konzentrationen zur Apoptose führen (39, 63).

Die Expression der kodierenden Gene für AR, BGT1, SMIT und HSP70 wird durch den gemeinsamen Transkriptionsfaktor tonicity-responsive enhancer binding protein/nuclear factor in activated T-cells 5 (TonEBP/NFAT5) reguliert (21, 66, 72, 74). Die entsprechenden Promotorregionen der jeweiligen Gene enthalten das *cis*-Element tonicity-responsive enhancer (TonE). Nach Bindung von TonEBP/NFAT5 an dieses Sequenzmotiv wird die Transkription der entsprechenden Gene initiiert (35, 45, 66, 74). Hierfür besitzt das als Homodimer vorliegende TonEBP/NFAT5 die für Transkriptionsfaktoren der *Rel*-Familie typische DNA-Bindungsdomäne. Erhöhte extrazelluläre Osmolalitäten bewirken eine vermehrte Expression von TonEBP/NFAT5 sowie dessen Phosphorylierung und Translokation vom Zytosol in den Zellkern, wo die Bindung an TonE-Elemente erfolgt (9, 17, 56). Im Tiermodell führt eine reduzierte TonEBP/NFAT5-Expression zu einer verminderten Expression der osmoprotektiven Gene AR, BGT1 und SMIT und geht mit einer

schwerwiegenden Schädigung medullärer Zellen sowie einem renalen Funktionsverlust einher (40).

1. 2. COX-2

Neben der Expression osmoprotektiver Gene und HSPs spielen das Enzym COX-2 und deren Produkte wichtige Rollen bei der Anpassung der Nierenmarkszellen an hohe interstitielle Osmolalitäten. Die medulläre COX-2-Expression korreliert mit der Urinosmolalität und ein Anstieg der interstitiellen Tonizität bewirkt eine Induktion der COX-2 auf mRNA- und Proteinebene (79).

Cyclooxygenasen sind membranständige Proteine. Es existieren zwei Isoformen mit ähnlicher Primärstruktur und katalytischer Aktivität, die COX-1 und die COX-2. Beide Isoenzyme sind sowohl am endoplasmatischen Retikulum als auch in der Kernmembran lokalisiert, wobei die COX-2 vor allem an letzterer Lokalisation nachgewiesen werden kann (50).

Die COX-2 katalysiert den Großteil der im Nierenmark produzierten Prostaglandine (79). Die Synthese der Prostaglandine erfolgt in zwei Abschnitten. Nach Freisetzung von Arachidonsäure aus Membranphospholipiden durch die zytosolische Phospholipase A_2 ($cPLA_2$), katalysiert die Cyclooxygenase die Synthese von Prostaglandin H_2 (PGH_2) aus Arachidonat. Das katalytische Zentrum der COX besteht aus einer Prostaglandin G–Synthase und einer Prostaglandin H–Synthase. Arachidonsäure wird dabei zunächst in einer Cyclooxygenase-Reaktion in Prostaglandin G_2 (PGG_2) und durch anschließende Peroxidase-Reaktion in PGH_2

umgewandelt (68). In weiteren Schritten werden die unterschiedlichen biologisch aktiven Prostaglandine aus PGH_2 synthetisiert (51).

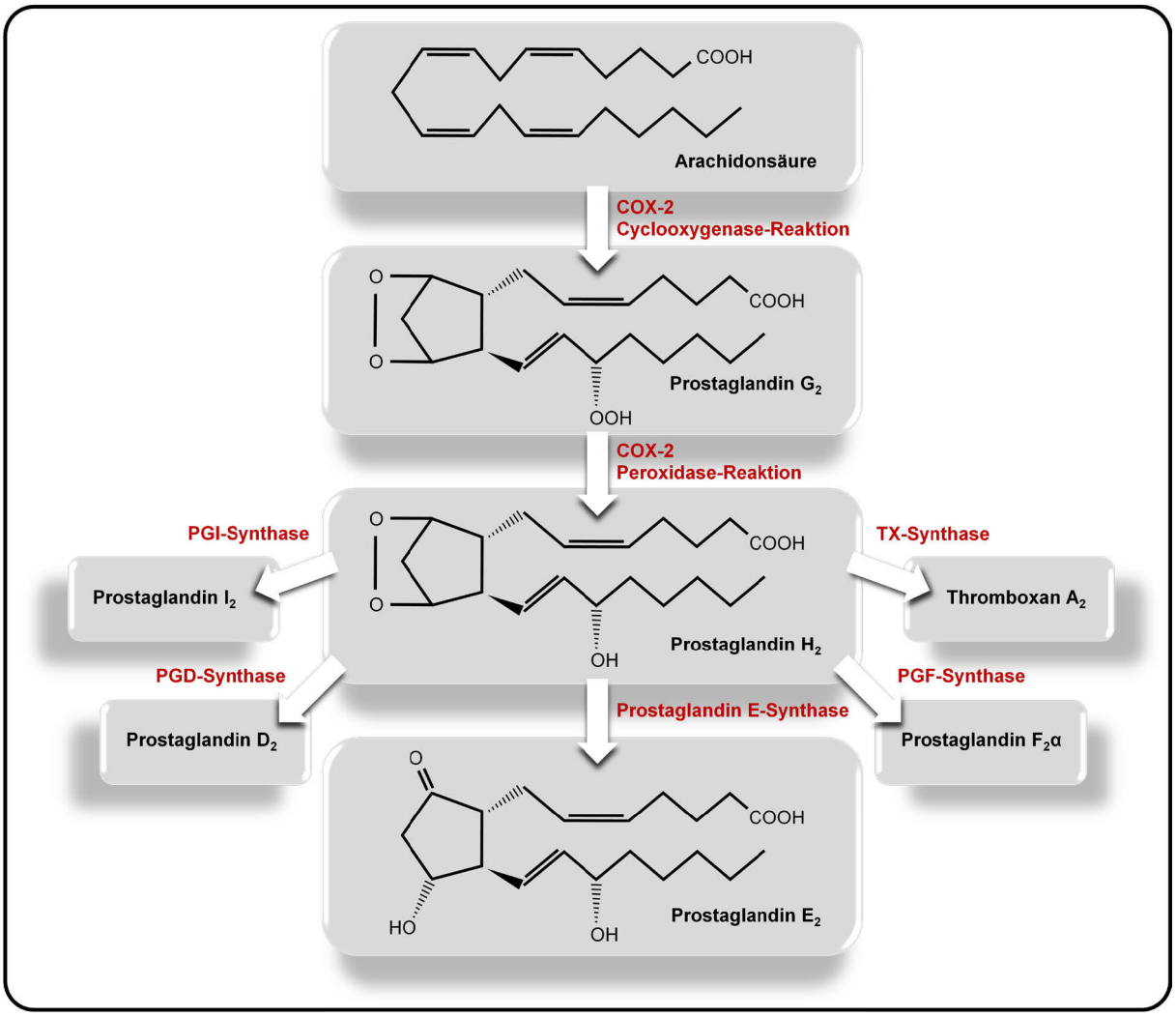

Abbildung 1. Biosynthese der Prostaglandine (51).

Während die COX-1 im Organismus ubiquitär konstitutiv exprimiert wird und eine Homöostase-Funktion bei der Prostaglandinsynthese wahrnimmt, ist die COX-2 durch diverse Stimuli induzierbar. Allerdings wird, im Unterschied zu den meisten anderen Geweben, im Nierenmark auch die COX-2 konstitutiv exprimiert und kann in Antidiurese zusätzlich induziert werden (16, 78, 79, 80).

Entsprechend dieser Induzierbarkeit des Enzyms enthält das COX-2-Gen im Gegensatz zum COX-1-Gen verschiedene transkriptionsregulierende Sequenzen wie κB-Motive und das cAMP-response element (CRE) über die eine Expression der COX-2 initiiert werden kann (36). Obwohl die osmoprotektiven Gene für AR, BGT1, SMIT und HSP70 durch den Transkriptionsfaktor TonEBP/NFAT5 reguliert werden, ist die COX-2-Transkription nicht TonEBP/NFAT5-abhängig. Transkriptionsfördernd auf die COX-2 wirken die Transkriptionsfaktoren nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) und das cAMP-resonse element binding protein (CREB), wobei der klassische Induktionsweg im Rahmen entzündlicher Prozesse vorwiegend durch NF-κB vermittelt wird (75, 78).

CREB ist ein über die cyclisches Adenosinmonophosphat (cAMP)-Signalkaskade induzierbarer Transkriptionsfaktor und gehört zur Klasse der Leucine Zipper. Seine basic leucine zipper (bZIP)-Domäne vermittelt eine Homodimerisierung und die DNA-Bindung (20). CREB bindet an das *cis*-Element CRE im COX-2-Promotor. CRE enthält 8 Basenpaare mit der Sequenz 5'-TGACGTCA-3' und ist üblicherweise innerhalb von 100 Nukleotiden stromaufwärts der TATA-box lokalisiert (14, 48). CREB wird über Phosphorylierung in seiner Kinase-induzierbaren Domäne (KID) aktiviert. Dabei erfolgt die Phosphorylierung des Serinrestes an Position 133 der KID durch die cAMP-abhängige Proteinkinase A (PKA) (22). Der PKA-Kinasekomplex besteht aus einem Tetramer zweier regulatorischer und zweier katalytischer Untereinheiten. Nach Aktivierung durch Bindung von cAMP lösen sich beide katalytischen C-Untereinheiten und translozieren passiv in den Zellkern (26). Hier aktivieren den Transkriptionsfaktor CREB durch Phosphorylierung. Anschließend können die Koaktivatoren CREB-binding-protein (CBP) und p300 über ihre KID-interagierende Domäne KIX den phosphorylierten Serinrest 133 von CREB

komplexieren (44, 61). Durch Bindung der Koaktivatoren wird die Transkription des CREB-Zielgens COX-2 initialisiert. CBP und p300 interagieren über Rekrutierung der RNA-Polymerase II mit der basalen Transkriptionsmaschinerie und initiieren eine Interaktion mit dem CRE-gebundenen Transkriptionsfaktor CREB (33, 38). Zudem besitzen CBP und p300 Histonacetylase-Aktivität, welche durch Acetylierung von Histonen die Chromatinstruktur für eine Bindung der basalen Transkriptions-maschinerie zugänglich macht (1, 59).

Die Intensität des extrazellulären cAMP-Stimulus korreliert hierbei mit der Menge der in den Zellkern translozierenden C-Untereinheiten der PKA und ist proportional zur resultierenden CREB-Aktivität. Reduzierte Signallevel bewirken eine verminderte CREB-Phosphorylierung und eine Abnahme der Zielgenexpression (25, 73).

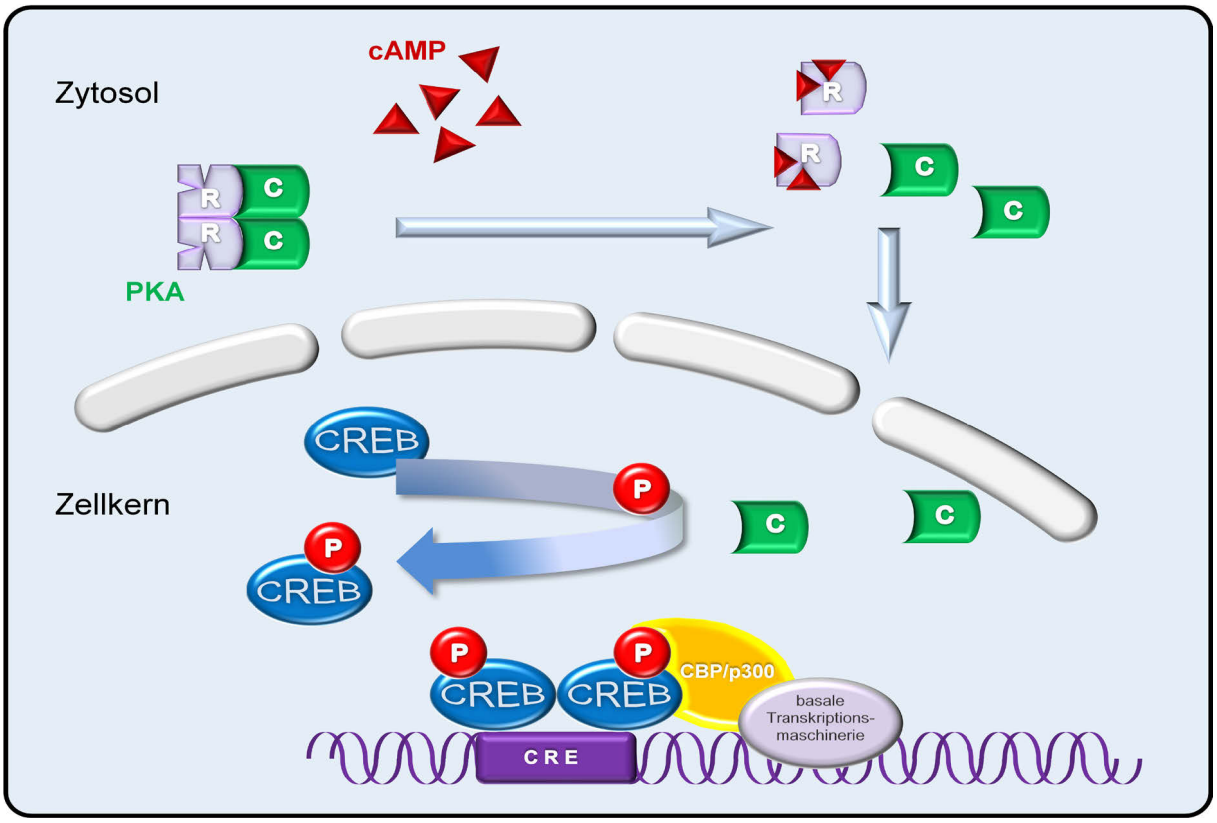

Abbildung 2. cAMP-abhängige CREB-Signalkaskade.

1. 3. Prostaglandin E_2

Prostaglandin E_2 (PGE_2), welches durch die Prostaglandin E-Synthase gebildet wird, ist das im Nierenmark quantitativ am stärksten produzierte Prostaglandin (4). PGE_2 wirkt auf auto- und parakrinem Weg nach Bindung an membranständige, G-Protein-gekoppelte E-Prostanoid-Rezeptoren (EP). Es existieren 4 verschiedene Subtypen (EP1-4) mit unterschiedlichen intrazellulären Signalkaskaden. Eine EP1-Stimulation bedingt eine intrazelluläre Calcium-Mobilisation durch Aktivierung der Phospholipase C, welche die Hydrolyse von Phosphatidylinositol-4,5-bisphosphat (PIP_2) zu Inositol-1,4,5-trisphosphat (IP_3) katalysiert. Durch Bindung von IP_3 an die IP_3-Rezeptoren des Endoplasmatischen Retikulums werden Calciumionen aus diesem intrazellulären Speicher freigesetzt. Diskutiert wird auch ein weiterer Phospholipase C-unabhängiger Signalweg, wonach ein extrazellulärer Calciumeinstrom die intrazelluläre Calciumkonzentration erhöht. Der EP3-Rezeptor hemmt über ein inhibitorisches G-Protein die intrazelluläre Bildung von cAMP. EP2 und EP4 sind G_s-Protein-gekoppelte Rezeptoren. Ihre Aktivierung durch PGE_2 führt über ein stimulierendes G-Protein und die Adenylatcyclase zu einer erhöhten intrazellulären cAMP-Konzentration (13, 28, 32, 51, 57, 71). Die Bindung von PGE_2 an den EP2-Rezeptor bewirkt über dessen Konformationsänderung die Aktivierung des gekoppelten G-Protein-Trimers. Nach Austausch von GDP zu GTP dissoziiert die α-Untereinheit vom βγ-Komplex und induziert die membrangebundene Adenylatcyclase, welche die Reaktion von ATP zu cAMP katalysiert (81). cAMP bindet mit hoher Affinität an die regulatorischen Untereinheiten der Proteinkinase A (PKA), woraufhin, wie zuvor beschrieben, sich beide katalytischen C-Untereinheiten vom Kinasekomplex lösen und nach Translokation in den Zellkern verschiedene

Transkriptionsfaktoren durch Phosphorylierung aktivieren.

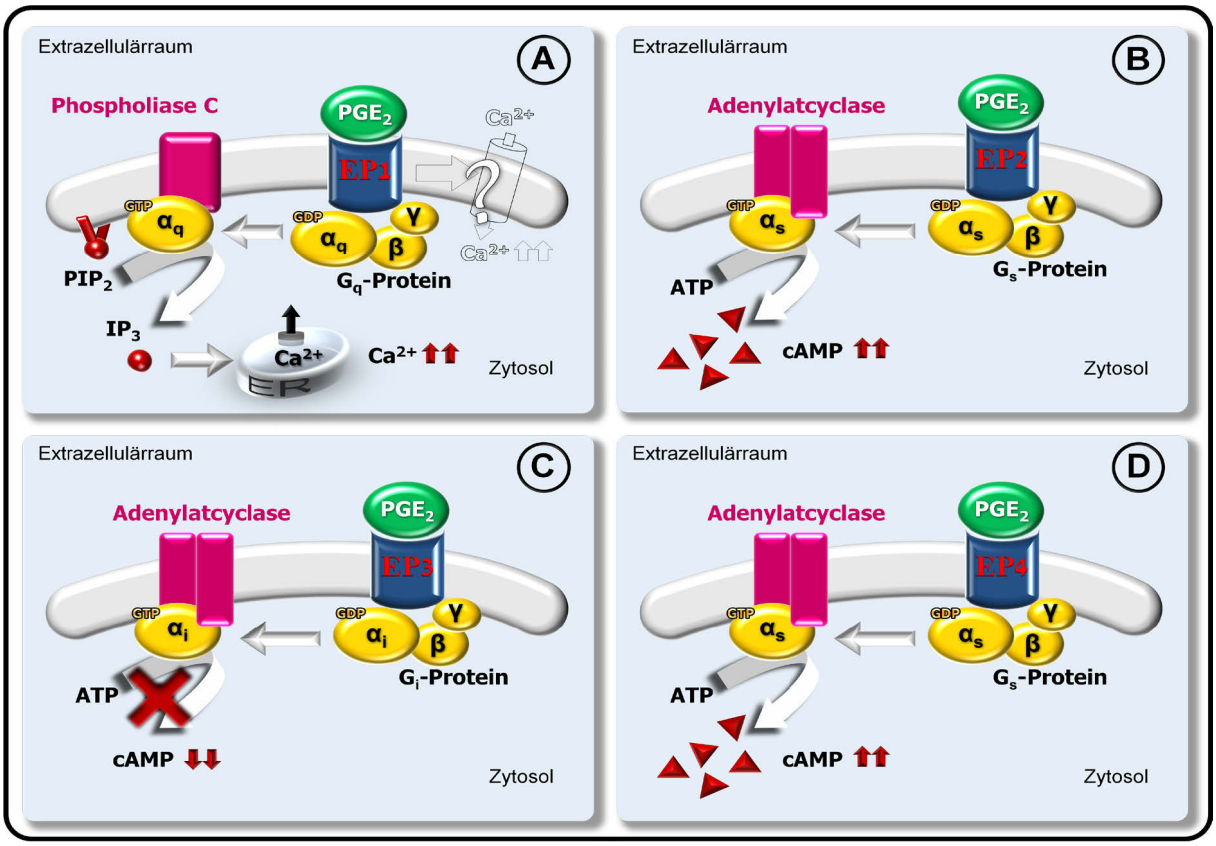

Abbildung 3. Signaltransduktion durch die Prostaglandin E_2-Rezeptoren EP1 (A), EP2 (B), EP3 (C) und EP4 (D)

Die vier Prostaglandinrezeptor-Subtypen unterscheiden sich nicht nur hinsichtlich ihrer Signaltransduktionswege, sondern auch bezüglich ihrer Rolle in der Regulation der Nierenfunktion und dementsprechend in ihrer Lokalisation im renalen Zellverband (5, 6). Die definitive Verteilung des EP2-Rezeptors ist noch nicht mit Sicherheit geklärt. Dieser konnte aber im Nierengewebe verschiedener Spezies in der äußeren und inneren Medulla nachgewiesen werden (24, 30, 49). Auch Madin-Darby canine kidney (MDCK) Zellen als Modell medullärer Sammelrohrzellen besitzen die Rezeptoren EP2 und EP4 (41, 58). Da EP2-defiziente Mäuse einen salz-sensitiven

arteriellen Hypertonus entwickeln, wird dem EP2-Rezeptor eine Bedeutung in der Regulation des Salzhaushaltes und der Blutdruckregulierung zugesprochen (34).

PGE_2 ist für eine normale Nierenfunktion essentiell. Es reguliert die Salz- und Wasserreabsorption im medullären dicken aufsteigenden Teil der Henle Schleife (mTAL) und im Sammelrohr durch die Inhibition entsprechender Kanäle (29, 31). Ferner vermittelt PGE_2 die Dilatation der absteigendenen Vasa recta und wirkt somit Vasokonstriktoren wie Angiotensin II, Vasopressin und Katecholaminen lokal entgegen um die Zellen des Nierenmarkes vor hypoxischer Schädigung zu bewahren (52, 54, 60). Durch Steigerung der Expression osmoprotektiver Gene fördert PGE_2 die Anpassung medullärer Zellen an die in dieser Nierenregion vorherrschenden hohen Osmolalitäten (46, 54, 55). Des Weiteren konnten PGE_2-vermittelte anti-apoptotische Effekte für verschiedene Zelltypen gezeigt werden (2, 11, 27, 37, 70). Der Apoptose-inhibierende Mechanismus wurde als EP2- und EP4-Rezeptor-abhängiger Signalweg in humanen pulmonalen Fibroblasten, in γ-Strahlung ausgesetzten Kryptenzellen des proximalen Jejunums von Mäusen und in UVB-bestrahlten murinen Hautzellen beschrieben. Letzteres wird über eine EP2-PKA-abhängige Phosphorylierung und Inaktivierung des proapoptotischen Proteins Bad durch PGE_2 vermittelt (11, 27, 70).

1. 4. Das Protein Bad und Apoptoseregulation

Bcl-2-associated death promotor (Bad) gehört zur B-cell lymphoma-2 (Bcl-2)-Protein-Familie und besitzt eine proapoptotische Funktion. Zur Bcl-2-Gruppe gehören

homologe Proteine mit antiapoptotischem (Bcl-2, Bcl-x_L, Mcl-1, A1) und pro-apotischem (Bad, Bax, Bak, Bcl-x_s) Effekt. Diese sind durch ein bis vier Bereiche homologer Aminosäuresequenzen, den sogenannten Bcl-2 homology (BH)-Domänen, gekennzeichnet (65). Durch Heterodimerbildung unter den einzelnen Mitgliedern modulieren diese gegenseitig ihre Funktion und regulieren den mitochondrialen Apoptoseweg. Der BH3-Domäne wird eine wichtige Rolle bei der Apoptoseeinleitung zugeschrieben. Diese kann bei allen proapoptotischen Mitgliedern nachgewiesen werden (10, 15, 23).

Das BH3-only-Protein Bad besitzt lediglich eine Sequenzhomologie in der BH3-Domäne. Im unphosphorylierten Zustand ist Bad in der äußeren Mitochondrien-membran lokalisiert. Die Regulation seiner Funktion erfolgt über Phosphorylierung an verschiedenen Serinresten, die durch extrazelluläre Faktoren wie Insulin-like Growth Factor-1 (IGF-1), Nerve Growth Factor (NGF), Platelet Derived Growth Factor (PDGF) und Interleukin-3 (IL-3) induziert werden kann (18). Unphosphoryliertes Bad bildet über die BH3-Domäne Heterodimere mit Bcl-x_L und hemmt damit dessen antiapototische Funktion (77). Dies bewirkt eine Freisetzung von Cytochrom C aus den Mitochondrien und Aktivierung der Caspase-Kaskade (64). Die durch die Proteinkinase A vermittelte Phosphorylierung des Serinrest an Position 155 (Ser^{155}) verhindert die Bindung der BH3-Domäne von Bad an Bcl-x_L und blockiert damit die inhibitorische Funktion von Bad auf das antiapoptotische Bcl-x_L. Die Phosphorylierung an Ser^{155} scheint somit durch Unterbrechung der intrinsischen Apoptosekaskade eine wichtige Rolle für das Zellüberleben zu spielen (19, 82).

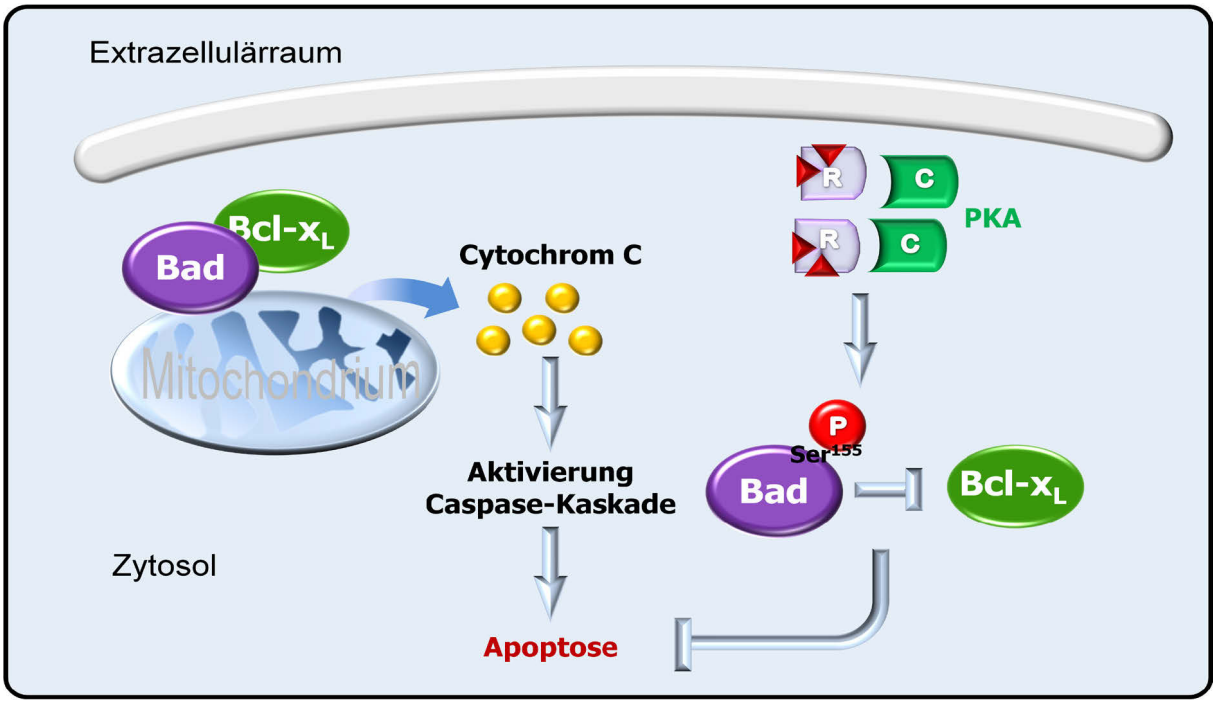

Abbildung 4. Das Protein Bad und Apoptoseregulation (19, 82).

1. 5. Zusammenfassung der vorliegenden Arbeiten

Die Zellen des Nierenmarkes sind vor allem in Antidiurese extrem lebensfeindlichen Umgebungsbedingungen ausgesetzt. In dieser Nierenregion herrschen nicht nur hohe interstitielle Osmolalitäten vor, sondern es erfolgen auch rasche und extreme Änderungen der Tonizität beim Übergang von Diurese in Antidiurese und umgekehrt. Durch diverse Strategien sichern medulläre Zellen nicht nur ihr Überleben, sondern wahren zudem ihre physiologische Funktion. Bei diesem Prozess scheinen eine rasche Induktion der COX-2 und die Bildung von PGE_2 von entscheidender Bedeutung zu sein. Demzufolge kann, insbesondere im dehydrierten Zustand eines Organismus, eine pharmakologische Hemmung der COX-2 durch unspezifische nicht-steroidale Antiphlogistika oder selektive COX-2-Inhibitoren eine renale Gewebeschädigung oder sogar ein akutes Nierenversagen verursachen.

Gegenstand der beiden vorliegenden Arbeiten war die Untersuchung der Rolle von COX-2 und PGE_2 bei der Osmoadaptation der Zellen des Nierenmarkes sowie deren Einfluss auf das Überleben dieser Zellen bei osmotischem Stress. Ferner sollten die zugrunde liegenden Signaltransduktionswege charakterisiert werden.

In der ersten Arbeit konnte gezeigt werden, dass PGE_2 in kultivierten MDCK-Zellen - hier als Modellsystem medullärer Sammelrohrzellen gewählt - das Zellüberleben bei einem Anstieg der Umgebungsosmolalitäten maßgeblich begünstigt. Damit einhergehend war eine verminderte Aktivität des Apoptosemarkers Caspase-3 nachweisbar. Unter hypertonen Bedingungen erfolgte eine Induktion der die Osmolyt-Akkumulation regulierenden Gene AR, SMIT und BGT1 zeitlich deutlich

nach der Induktion von HSP70 und COX-2. Dies deutete darauf hin, dass die intrazelluläre Akkumulation signifikanter Mengen an Osmolyten in dieser frühen Phase des osmotischen Stresses nicht maßgebend für Zellüberleben ist. Mutmaßlich spielen HSP70 und COX-2 zu diesem Zeitpunkt eine entscheidendere Rolle. In Gegenwart von exogen zugeführtem PGE_2 waren die bereits durch osmotischen Stress gesteigerten mRNA-Level der osmoprotektiven Gene AR, SMIT, HSP70 und COX-2 nochmals signifikant gesteigert. Es ließ sich eine deutliche Stimulation durch PGE_2 sowohl auf die AR-Promoter-Aktivität als auch auf die Expression von Aldose-Reduktase nachweisen. Es zeigte sich allerdings kein Einfluss von PGE_2 auf das quantitative Vorkommen des Transkriptionsfaktors TonEBP/NFAT5, welcher die Expression von AR, SMIT und HSP70 reguliert oder auf eine Ton-E-gesteuerte Promotoraktivität. Es war daher anzunehmen, dass der beobachtete PGE_2-Effekt nicht durch eine gesteigerte transkriptionelle Aktivität von TonEBP/NFAT5 vermittelt wird.

Interessanterweise konnte bei osmotischem Stress ebenfalls eine zusätzliche stimulierende Wirkung von PGE_2 auf die COX-2-Expression selbst demonstriert werden. Diese Beobachtung deutete auf das Vorhandensein eines positiven Feedback-Mechanismus hin. Nach Aktivierung des Harnkonzentrierungs-mechanismus in vivo beobachtet man die Produktion großer Mengen PGE_2 in der renalen Medulla. Dies könnte durch den im Rahmen der vorliegenden Arbeit identifizierten positiven Rückkopplungsmechanismus von PGE_2 auf die COX-2-Expression begünstigt werden.

Die zweite Arbeit untersuchte weiterführend diesen PGE_2-vermittelten positiven Rückkopplungsmechanismus auf die COX-2-Expression und charakterisierte den

zugrunde liegenden Signaltransduktionsweg sowie dessen funktionelle Relevanz auf das Zellüberleben bei osmotischem Stress. Der stimulierende PGE_2-Feedback-Effekt auf die COX-2-Expression, der unter isotonen Bedingungen nicht zu beobachten war, ließ sich unter hyperosmolaren Bedingungen auf mRNA- und Proteinebene sowie durch eine gesteigerte COX-2-Enzymaktivität nachweisen. Die G_s-Protein-Kopplung der Prostaglandinrezeptoren und die Tatsache, dass der COX-2-Promotor ein funktionelles CRE enthält, ließen vermuten, dass der stimulierende Effekt von PGE_2 durch einen cAMP-PKA-CREB-abhängigen Signalweg vermittelt wird. Durch verschiedene Versuche mit pharmakologischen Agonisten und Inhibitoren konnte ein PGE_2-vermittelter Signalweg demonstriert werden, der über den G_s-Protein-gekoppelten Prostaglandinrezeptor EP2 zu einem Anstieg der intrazellulärem cAMP-Konzentration und Aktivierung der Proteinkinase A führt. Passend zu dieser Beobachtung zeigte sich der Effekt von PGE_2 ebenfalls in den nachgeschalteten Signalwegen in einer (i) Aktivierung von CREB mit (ii) nachfolgender Transkription von COX-2-mRNA sowie einer (iii) gesteigerten CRE-abhängigen Reportergen-Aktivität und (iv) Stimulation der COX-2-Promotor-Aktivität über ein CRE. Auch war nach gezielter Mutagenese der mutmaßlich verantwortlichen CRE-Sequenz in einem COX-2-Promotor-Reporterkonstrukt der stimulierende Effekt von PGE_2 auf dessen Aktivität nicht mehr nachzuweisen.

Ferner ließ sich ein potenziell überlebensfördernder PGE_2-abhängiger Mechanismus demonstrieren. In Gegenwart von extern zugeführtem PGE_2 zeigten sich ein verbessertes Zellüberleben sowie eine verminderte Aktivität des biochemischen Apoptose-Indikators Caspase-3. Es konnte ein EP_2-cAMP-abhängiger Signalweg identifiziert werden, der über eine PKA vermittelte Phosphorylierung des proapoptotischen Proteins Bad am Serinrest 155 zu dessen Inaktivierung führt.

Durch pharmokologische Unterbrechung dieses Signalweges kam es zur Aufhebung des positiven PGE_2-Effekts auf das Zellüberleben und zur Aktivierung der Caspase-Kaskade mit erhöhter Caspase-3-Aktivität bei osmotischem Stress.

Zusammenfassend deuteten die Ergebnisse auf einen EP_2-cAMP-PKA-CREB-abhängigen Signalweg hin, welcher durch PGE_2 eine gesteigerte COX-2-Expression im Sinne eines positiven Rückkopplungseffektes bei osmotischem Stress vermittelt. PGE_2 förderte zusätzlich die Expression anderer osmoprotektiver Gene. Zudem zeigte sich ein antiapoptotischer Effekt durch einen PGE_2-EP_2-cAMP-PKA-abhängigen Mechanismus, der zur Phosphorylierung und damit Inaktivierung des pro-apoptotischen Proteins Bad führt. Der hier charakterisierte PGE_2-Signalweg hat funktionelle Bedeutung für die Anpassung der Nierenmarkszellen an ihre lebensfeindlichen Umweltbedingungen, ihr Zellüberleben und die Wahrung ihrer physiologischen Funktion bei osmotischem Stress. Eine pathophysiologische Bedeutung bei der Entstehung der durch nicht-steroidale anti-inflammatorische Medikamente (NSAID) induzierten Nierenschädigung ist zudem zwanglos vorstellbar.

Beitrag des Doktoranden zu der in Ko-Autorenschaft publizierten Arbeit:

Das Konzept der Publikation wurde von PD Dr. Wolfgang Neuhofer ausgearbeitet. Die Durchführung und Auswertung der Experimente erfolgte in Zusammenarbeit mit Daniela Steinert.

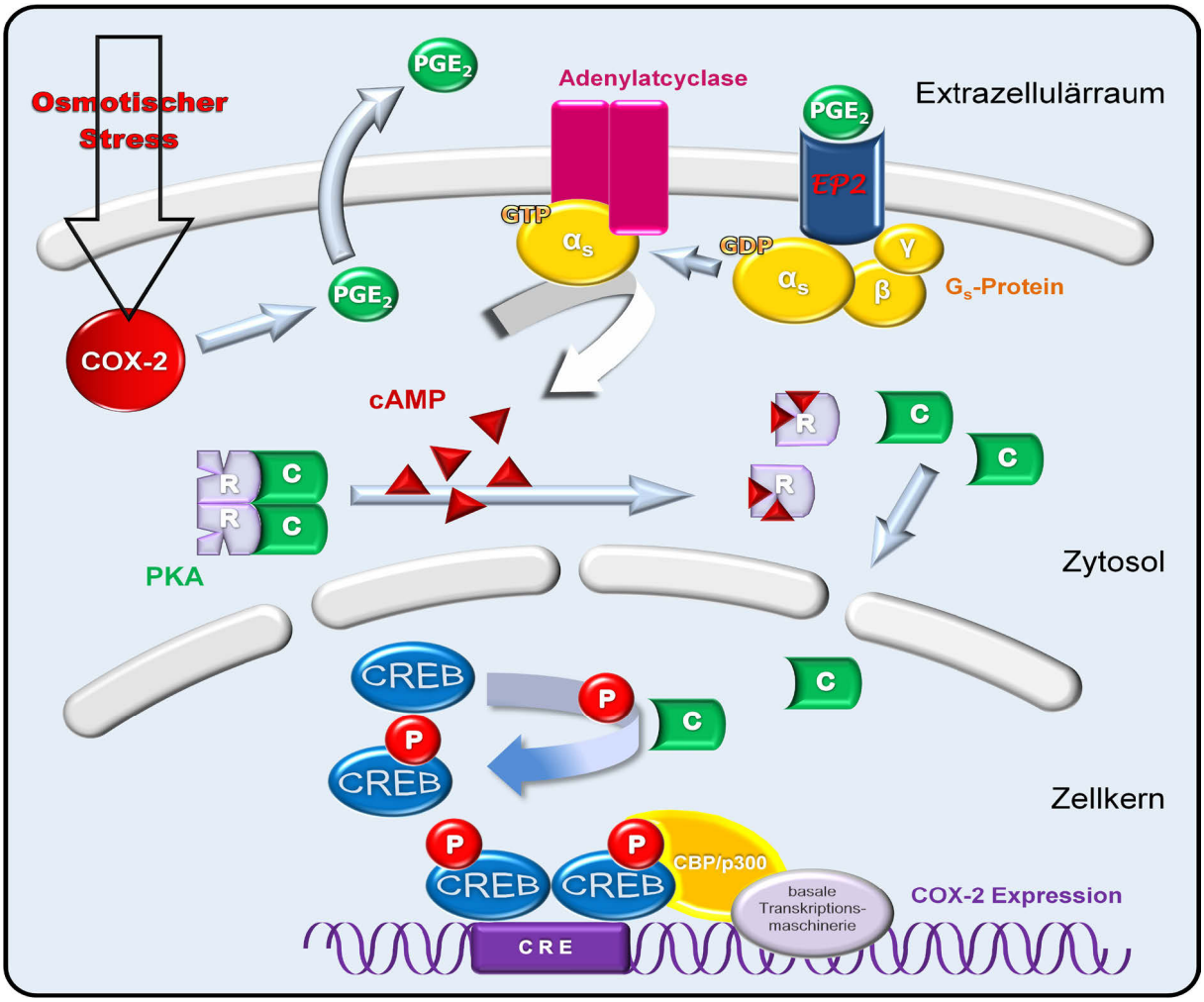

Abbildung 5 . Positiver Rückkopplungseffekt der COX-2 durch PGE_2.

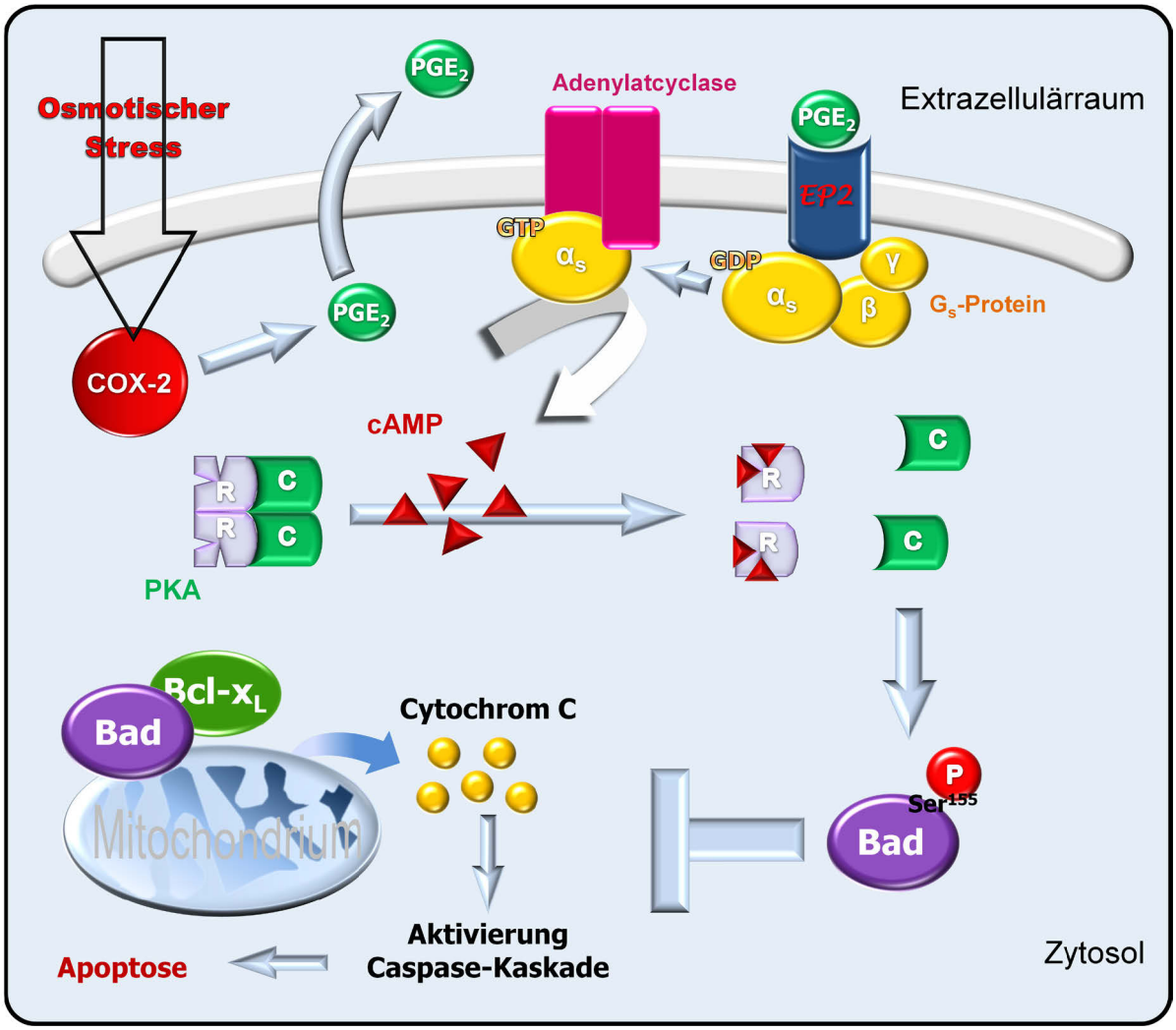

Abbildung 6. Antiapoptotischer Effekt durch PGE_2-abhängigen Mechanismus.

1. 6. Summary of the present publications

The interstitial environment of the renal medulla is characterized by extremely high osmolalities in antidiuesis. And the resident cells are exposed to a rapid increase in interstitial tonicity following stimulation of the urinary concentration mechanism. The renal medullary cells employ several osmoadaptive strategies to promote cell survival and maintain their proper function, thereby preserving renal integrity. Rapid induction of COX-2 and subsequent formation of PGE_2 plays a critical role in this process. Pharmacological enzyme inhibition by non-steroidal anti-inflammatory drugs that target COX-2 selectively or non-selectively is known to be associated with structural and functional renal damage.

The present studies analyzed the role of COX-2 and PGE_2 in osmoadaptation of renal medullary cells and addressed their effects on cell survival in the hostile environmental of the renal medulla. A further focus was to delineate the underlying signaling pathways.

The first section dealt with the role of COX-2 and PGE_2 in osmoadaptation. Madin-Darby canine kidney (MDCK) cells were used as a cell culture model for renal collecting duct cells and were exposed to osmotic stress. The presence of exogenous PGE_2 promoted cell survival significantly under these conditions and substantially attenuated caspase-3-activity, a biochemical index of apoptosis. In addition tonicity-induced up-regulation of mRNA abundance of the osmosensitive genes AR, SMIT, HSP70 and COX-2 were further potentiated in presence of PGE_2. The induction of HSP70 and COX-2 preceded that of genes that are involved in the

intracellular accumulation of organic osmolytes (AR, BGT1, and SMIT). This is consistent with the assumption that in the early phase of osmotic stress cell survival and function does not require the accumulation of significant amounts of organic osmolytes. However it appears to depend on the induction of HSP70 and COX-2. PGE_2 further increased the activity of an AR-promoter-driven reporter construct and stimulated the expression of AR under hypertonic conditions. Surprisingly, PGE_2 had no detectable effect on either the abundance of the transcriptional factor TonEBP/NFAT5 that stimulates the expression of AR, SMIT and HSP70, or on TonE-driven promoter activity. These findings indicated that the stimulatory effect of PGE_2 is not mediated by increased TonEBP/NFAT5 activity.

In addition PGE_2 also increased the expression of COX-2 during osmotic stress, suggesting the existence of a positive feedback mechanism. In vivo the induction of the urinary concentration mechanism and subsequent increase in interstitial tonicity in the renal medulla cause rapid production of large amounts of prostanoids. This ability of renal medullary cells may reflect the observed positive feedback loop of PGE_2 on COX-2.

The second section extended the previous observation that PGE_2 increases COX-2 expression in a positive feedback loop and focused on the underlying signaling pathway and its functional relevance. The stimulatory effect of PGE_2 on COX-2 abundance was not evident under isotonic conditions but could be demonstrated under osmotic stress at the mRNA and protein level, and by elevated COX-2 enzyme activity. The coupling of the prostanoid receptors to G_s-proteins and the existence of a CRE in the COX-2 promoter region suggested a corresponding signaling pathway. On the basis of pharmacological agonist- and inhibitor-based experiments, a PGE_2-

mediated signaling pathway was identified and involved the G_s-protein-coupled prostaglandin receptor EP2, subsequent increase of intracellular cAMP levels and activation of PKA. The effect of PGE_2 was also consistently detectable in downstream signaling components. Activation of CREB with consequent increased transcription of COX-2-mRNA, enhanced CRE-driven reporter activity and stimulation of COX-2-promotor driven reporter activity by CRE-signaling could be demonstrated. The stimulatory effect of PGE_2 on COX-2-promoter activity was abolished after site-directed mutagenesis of the putative CRE-element. Additionally, a PGE_2-EP2-cAMP-PKA-mediated survival mechanism was detected. Phosphorylation of the proapoptotic protein Bad at serine residue 155 by PGE_2-activated PKA, and hence inactivation of Bad, promoted cell survival and reduced caspase-3-activity. Pharmacological disruption of this pathway abolished the positive effect of PGE_2 on cell survival and induced caspase activation under hypertonic conditions.

In conclusion this study identified an EP2-cAMP-PKA-CREB-dependent signaling pathway that mediates a positive feedback mechanism of the major medullary prostaglandin PGE_2 on COX-2 expression in response to osmotic stress. In addition PGE_2 potentiated the expression of other osmoprotective genes. The study further demonstrated that PGE_2 improved cell survival under hypertonic conditions substantially by EP2-cAMP-PKA-signaling via phosphorylation and inactivation of the proapoptotic protein Bad. The PGE_2-dependent signaling pathway characterized here has functional importance for the adaptation of renal medullary cells to their hostile environment, for providing cell survival, and maintaining cell integrity. Pathophysiological relevance for the development of renal injury induced by non-steroidal anti-inflammatory drugs (NSAID) appears conceivable.

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2. Ergebnisse

2. 1. Prostaglandin E_2 stimulates expression of osmoprotective genes in MDCK cells and promotes survival under hypertonic conditions

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Prostaglandin E_2 stimulates expression of osmoprotective genes in MDCK cells and promotes survival under hypertonic conditions

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Running title: PGE_2 and osmoadaptation

Key words: kidney, osmoadaptation, prostaglandin E_2

Abstract

The cells of the renal medulla produce large amounts of prostaglandin E_2 (PGE_2) via cyclooxygenases (COX)-1 and -2. PGE_2 is well known to play a critical role in salt and water balance and maintenance of medullary blood flow. Since renal medullary PGE_2 production increases in antidiuresis, and since COX inhibition is associated with damage to the renal medulla during water deprivation, PGE_2 may promote the adaptation of renal papillary cells to high interstitial solute concentrations. To address this question, MDCK cells were exposed to a gradual tonicity increase in the presence or absence of 20 µm PGE_2 prior to analysis of (i) cell survival, (ii) expression of osmoprotective genes (AR, BGT1, SMIT, HSP70 and COX-2), (iii) subcellular TonEBP / NFAT5 abundance, (iv) TonEBP / NFAT5 transcriptional activity and (v) aldose reductase promoter activity. Cell survival and apoptotic indices after raising the medium tonicity improved markedly in the presence of PGE_2. PGE_2 significantly increased tonicity-mediated up-regulation of AR, SMIT and HSP70 mRNAs. However, neither nuclear abundance nor TonEBP / NFAT5-driven reporter activity were elevated by PGE_2, but aldose reductase promoter activity was significantly increased by PGE_2. Interestingly, tonicity-induced COX-2 expression and activity was also stimulated by PGE_2, suggesting the existence of a positive feedback loop. These results demonstrate that the major medullary prostanoid, PGE_2, stimulates the expression of osmoprotective genes and favours the adaptation of medullary cells to increasing interstitial tonicities, an effect that is not explained directly by the presence of TonEs in the promoter region of the respective target genes. These findings may be relevant in the pathophysiology of medullary damage associated with analgesic drugs.

Introduction

During antidiuresis, the cells of the renal medulla are exposed to a uniquely harsh environment, characterized by extreme concentrations of NaCl and urea, in addition to low oxygen tension (Brezis & Rosen, 1995; Neuhofer & Beck, 2005). Furthermore, during transition from diuresis to antidiuresis and vice versa, these cells are challenged by massive changes in interstitial solute concentrations. After stimulation of the urinary concentrating mechanism, renal papillary interstitial tonicity may double within a few hours (Beck et al. 1992). Specifically, it has been demonstrated that following intravenous administration of lysine-vasopressin, papillary tissue sodium concentration increases from approximately 140 to more than 300 mM within 2 h (Atherton et al. 1970).

After tonicity increase, renal papillary cells initially shrink, thus leading to elevated concentration of intracellular electrolytes (i.e. the cellular ionic strength). The cells then activate volume regulatory mechanisms, which in turn allow restoration of cell volume, although intracellular ion concentrations remain elevated. A sustained rise in cellular ionic strength is, however, associated with DNA double-strand breaks and induces apoptosis in renal epithelial cells (Galloway et al. 1987; Kültz & Chakravarty, 2001). In the longer term, medullary cells achieve osmotic equilibrium with the high interstitial solute concentrations by intracellular accumulation of small, non-perturbing organic compounds i.e. compatible osmolytes, including myo-inositol, sorbitol and betaine (Burg et al. 1997; Neuhofer & Beck, 2005). The elevated intracellular concentration of these substances predominantly results from increased uptake from the extracellular space (myo-inositol, betaine) or from intracellular synthesis (sorbitol). The proteins involved in intracellular osmolyte accumulation include the

sodium-dependent myo-inositol cotransporter-1 (SMIT1), the sodium- and chloride-dependent betaine / GABA transporter-1 (BGT1), and aldose reductase (AR), which converts glucose to sorbitol. In addition, the expression of HSP70, a molecular chaperone expressed abundantly in the renal papilla (Cowley et al. 1995), is stimulated by tonicity stress and is believed to contribute to protection of medullary cells from the acute effects of hypertonicity (Neuhofer & Beck, 2005). The expression of the genes involved in osmolyte accumulation and that of HSP70 is stimulated by a common transcriptional activator, tonicity-responsive enhancer binding protein/nuclear factor of activated T cells-5 (TonEBP / NFAT5) (Woo et al. 2002a, b). The importance of this transcriptional activator for the cells of the renal medulla has been demonstrated in mice lacking TonEBP / NFAT5 (Lopez-Rodriguez et al. 2004). These animals show renal medullary atrophy as a result of reduced expression of tonicity-responsive genes (Lopez-Rodriguez et al. 2004).

The action of cyclooxygenases (COX) in the renal medulla contributes importantly to the adaptation of medullary cells to their hostile environment. It has been well documented that prostanoids are important modulators of both medullary perfusion and tubular solute reabsorption, thereby linking tubular work to oxygen availability (Navar et al. 1996; Pallone et al. 2003; Neuhofer & Beck, 2005). In agreement, during antidiuresis, the renal medulla produces large amounts of prostaglandin E_2 (PGE_2), most likely via COX-2, that is expressed constitutively in the renal medulla (Yang, 2003; Yang et al. 2005). COX inhibition by non-steroidal anti-inflammatory drugs (NSAID) has long been known to be associated with renal medullary injury, particularly in situations with stimulation of the renal concentrating mechanism (Schlöndorff, 1993; De Broe & Elseviers, 1998). Thus, the major medullary prostanoid, PGE_2, may promote the adaptation of papillary cells to increasing

interstitial solute concentrations. This study aimed to address the question of whether PGE_2 improves survival of medullary cells exposed to osmotic stress.

Methods

Cell culture and experimental protocol

Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC CCL 34). The cells were maintained under standard conditions in Dulbecco's modified Eagles' medium (low glucose) containing 10% fetal bovine serum (FBS) and 1% penicillin / streptomycin. Two days prior to the experiments, the cells were plated in 60 mm, 35 mm (Greiner, Frickenhausen, Germany), or 24-well plates (Nunc, Roskilde, Denmark) and grown to confluence. Subsequently, the medium tonicity was gradually increased over 2 h in eight steps (addition of 25 mosmol (kg H_2O)$^{-1}$ NaCl every 15 min; final medium osmolality 500 mosmol (kg H_2O)$^{-1}$). To elevate the medium tonicity to 700 mosmol (kg H_2O)$^{-1}$, osmolality was raised in 16 steps of 25 mosmol (kg H_2O)$^{-1}$ each by NaCl addition over 4 h. Tonicity increases were performed by adding the required volumes of a 4 m NaCl stock solution drop by drop to the dishes. Changes in pH and temperature as well as mechanical agitation were kept to a minimum. The isotonic control cells were treated accordingly, except that equal volumes of PBS were added to the cells, thus ruling out that the experimental procedure per se had negative effects on cell viability. Tonicity was increased either in the presence of 20 µm PGE_2 (Sigma, Schnelldorf, Germany; dissolved as a 20 mm stock solution in ethanol) or the same volume of vehicle alone. Subsequently, the cells were processed as indicated.

Northern blot analysis

Following the treatments, the cells were lysed by the addition of TRI reagent (1 ml per 60 mm dish; PeqLab, Erlangen, Germany) and total RNA was recovered

according to the manufacturer's recommendations. Aliquots of 20 µg were electrophoresed through 1% agarose–formaldehyde gels, subsequently blotted onto positively charged nylon membranes (Roche, Mannheim, Germany) and immobilized by UV crosslinking as described elsewhere (Neuhofer et al. 2002). Relative mRNA abundance of the respective genes was monitored by hybridization with the following digoxigenin-labelled cDNAs: human AR (GenBank accession number J05474), canine SMIT1 (M85068), canine BGT1 (M80403), human inducible HSP70 (M11717), bovine COX-2 (AF031698) and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; X01677). Generation of non-radioactive, digoxigenin-labelled probes, hybridization conditions and stringency washes are described in detail elsewhere (Neuhofer et al. 2002). Signals were subsequently quantified using Image J software (NIH, Bethesda, MA, USA) and the mRNA abundance of each individual gene was normalized to that of GAPDH to correct for differences in RNA loading.

Determination of TonEBP/NFAT5 cytosolic and nuclear abundance

Subcellular extracts were prepared with nuclear and cytosolic extraction reagents (NE-PER; Pierce, Rockford, IL, USA) according to the manufacturer's recommendations, with broad specificity protease inhibitor cocktail (Sigma) added at 1:100 (v/v). Aliquots of 10 µg from each fraction were run subsequently on 8% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking non-specific binding sites with blocking buffer (5% non-fat dry milk–0.1% Tween-20 in PBS) for 1 h, TonEBP / NFAT5 abundance was determined by incubation with TonEBP / NFAT5 antiserum (1:3000; Calbiochem, Darmstadt, Germany) in blocking buffer overnight at 4°C. Following washing with PBS–0.1% Tween-20 (3 x 5 min) and

incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (1: 10 000 in blocking buffer, 1 h incubation at room temperature), bands were visualized by enhanced chemiluminescence (Pierce) and analysed using Image J software.

Reporter gene assays

For assessment of TonEBP transcriptional activity, a reporter construct based on secreted alkaline phosphatase (SEAP) (Berger et al. 1988) was prepared. SEAP is a truncated form of human placental alkaline phosphatase lacking the membrane anchoring domain. Thus, the truncated enzyme is secreted efficiently into the culture supernatant and SEAP activity in the supernatant is directly proportional to SEAP mRNA and protein abundance (Berger et al. 1988; Cullen & Malim, 1992). For generation of a TonE-driven SEAP construct, a DNA fragment containing two copies of the human TonE sequence with adjacent *Kpn*I and *Bgl* II sites was prepared and inserted into the respective sites of pSEAP-control (Clontech, Mountain View, CA, USA), thereby adding the 2 x TonE sequence upstream from the SV40 early promoter. Subsequently, the 2 x TonE-SV40 early promoter fragment was released by *Kpn*I and *Nru*I and inserted into the respective sites of pSEAP-basic (Clontech). In the final construct, pSEAP-TonE, the SEAP-gene is preceded by two copies of the human TonE sequence and the SV40 early promoter, respectively (see also Fig. 6).

To assess the effect of PGE_2 in the context of a native promoter, a 1.5 kb fragment corresponding to nucleotides −1505 to +22 of the human aldose reductase promoter was amplified by PCR from genomic DNA from HEK 293 cells by PCR using primers 5′-AGCT-CGAGCAAACCAACAACAAAGCCTC-3′ (forward) and 5′-GCAAGCTTCGC GTACCTTTAAATAGCCC-3′ (reverse), which introduced *Xho*I and *Hind*III sites to 5′- and 3′- ends, respectively. The fragment was subsequently inserted into the

corresponding sites of pSEAP2-basic (Clontech). This region has been shown previously to confer osmotic inducibility and to contain at least one TonE (Ruepp et al. 1996; Nadkarni et al. 1999). The final construct, pSEAP-ARpro, was sequenced to verify orientation and sequence of the insert.

For stable transfection of MDCK cells with the TonE construct, exponentially growing cells were transfected (5 µg DNA per 60 mm dish) using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. As pSEAP-2xTonE lacks eukaryotic selection markers, the cells were cotransfected with a 20:1 molar ratio of pSEAP-2xTonE and pcDNA3.1 (containing a neomycin-resistance gene; Invitrogen, Karlsruhe, Germany). Following selection with 600 µg ml^{-1} geneticine, 40 stable clones were expanded and assessed for tonicity-inducible SEAP activity (see below). Several healthy clones with highly tonicity-inducible SEAP activity were subsequently pooled and used for experiments at passages 3–6 (in the following denoted as MDCK-pSEAP-TonE).

To assess the effect of PGE2 on TonEBP transcriptional activity under hypertonic conditions, MDCK-pSEAP-TonE cells were seeded in a 24-well plate 2 days prior to the experiment at a density to reach confluence within 2 days. Prior to the experiments, the medium (1 ml DMEM without phenol red lacking G418 per well) was refreshed and the cells were exposed to a gradual tonicity increase to 500 mosmol kg^{-1} as described above. After the indicated periods, 50 µl medium was removed and stored in 96-well plates at −80°C for further analysis. Prior to determination of SEAP activity, the 96-well plates containing the conditioned medium were sealed and incubated for 45 min in a water bath at 65°C to inactivate endogenous alkaline phosphatase activity, since SEAP is extremely heat stable (Cullen & Malim, 1992). Subsequently, 150 µl *p*-nitrophenyl phosphate liquid substrate (Sigma) was added

and the mixture incubated for 1–2 h at room temperature. Finally, SEAP activity was assessed at 405 nm in a microplate reader.

Transient transfections were performed in 24-well plates at 50–75% cell confluence with a 3:1 molar ratio of pSEAP-ARpro and pcDNA3-LacZ using Effectene transfection reagent according to the manufacturer's instructions. After 24 h, the cells were used for experiments, and SEAP activity was determined as described above and subsequently normalized to that of β-galactosidase. Briefly, cells were washed with PBS and lysed *in situ* by vigorous shaking for 5 min after the addition of 135 µl TBS–0.1% Triton X-100, followed by two freeze–thaw cycles. Then, 600 µl β-galactosidase substrate was added per well (0.1 mm chlorophenol red-beta-d-galactopyranoside (Roche, Mannheim, Germany) in 100 mm potassium phosphate buffer, pH 7.5, containing 2 mm DTT and 1 mm $MgCl_2$). The reaction was incubated for 2–4 h at room temperature and finally read at 574 nm.

Assessment of cell survival

After the respective treatments, detached cells were aspirated, adherent cells were collected by trypsinization, and cell numbers in both fractions were counted in a haemocytometer. The surviving fraction was expressed as the adherent/total (adherent plus detached) cell ratio as previously described (Neuhofer et al. 2001).

Determination of lactate dehydrogenase (LDH) release

Following the respective treatments, a small aliquot of medium was removed and adherent cells were then collected and disrupted by repeated vigorous passage through a 26-gauge needle. LDH activity was determined using a commercially available assay and expressed as the ratio between the activities in the supernatant

and total LDH activity (supernatant plus adherent) as previously described (Neuhofer et al. 2001).

Determination of caspase-3 activity

Caspase-3 activity was assessed using an assay based on the cleavage of a synthetic, *p*-nitroanilin-conjugated caspase-3 substrate (Asp-Glu-Val-Asp; CaspACE Assay System; Promega, Madison, WI, USA). Briefly, cells in 35-mm dishes were washed with ice-cold PBS after the indicated treatments and disrupted by the addition of 100 µl lysis buffer. After three freeze–thaw cycles, the lysates were cleared by centrifugation at 12000 g at 4°C for 20 min. Caspase-3 activity was monitored in 10 µl aliquots from the supernatant by an increase in absorbance at 405 nm according to the manufacturer's recommendations, and was referred subsequently to the protein concentration in the cell lysates. Relative caspase-3 activity is expressed as nanomoles of *p*-nitroanilin released per milligram of protein in 1 h.

Determination of COX activity

COX activity in lysates of MDCK cells was assessed using a commercially available assay (COX activity assay kit; Cayman Chemicals, Ann Arbor, MI, USA) based on the colourimetric measurement of the peroxidase activity of cyclooxygenase by monitoring the formation of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylendiamine (TMPD) at 620 nm. Briefly, cells were washed with ice-cold TBS and harvested in 200 µl TBS containing 1 mm EDTA and 1% (v/v) protease inhibitor cocktail (Sigma), and lysed mechanically by repeated vigorous passage through a 26-gauge needle. Subsequently, the lysate was cleared by centrifugation at 12000 g for 15 min at 4°C

and COX activity was assessed on equal volumes of the supernatant according to the recommendations of the manufacturer. To distinguish COX-2 from COX-1 activity, the samples were compared to a corresponding one containing an isoenzyme-specific inhibitor for COX-2 and COX-1, respectively (DuP-697, SC-560; 300 nm final concentration each). Subsequently, COX activity was normalized to the protein concentration in the lysates and expressed as U (µg of protein)$^{-1}$.

Measurement of intracellular electrolytes

MDCK cells were grown on collagen-coated filter membranes (Millicell-CM; Millipore, Bedford, MA, USA). At the end of the experiments, the medium was removed rapidly and the filters covered with a standard albumin solution applied on both apical and basal sides and snap frozen in a mixture of propane and isopentane (3:1 v/v; −196°C) as described elsewhere (Beck et al. 1992; Neuhofer et al. 2002). The standard albumin solution was prepared by dissolving 2 g bovine serum albumin in 10 ml of the respective medium. Cryosections (1 µm) were cut at −80°C using an ultracryomicrotome (LKB, Bromma, Sweden). The cryosections were then freeze-dried and analysed in a scanning electron microscope equipped with an X-ray detector system as described in detail elsewhere (Beck et al. 1992). Intracellular electrolyte concentration is expressed as mmol (kg wet weight)$^{-1}$.

Statistical analyses

Data are expressed as means ±s.e.m. The significance of differences between the means was assessed by Student's *t* test. $P < 0.05$ was regarded as significant.

Results

Effect of PGE_2 on survival of MDCK cells under hypertonic conditions

To determine whether PGE_2 enhances cell survival under osmotic stress, MDCK cells were subjected to a gradual tonicity increase to 700 mosmol (kg H_2O)$^{-1}$ by NaCl addition over a period of 4 h in the presence of 20 µm PGE_2 or only vehicle ethanol. Comparable tonicity increases have been observed in the papilla of the mammalian kidney *in vivo* 2–4 h following acute stimulation of the urinary concentrating mechanism (Atherton et al. 1971). As the interstitial tonicity rises continuously over several hours after stimulation of the urinary concentrating mechanism, in the present experiments a nearly linear osmolality increase was employed to mimic more closely the situation in the renal medulla *in situ* compared with a single step increase.

Since hypertonicity induces apoptosis in non-protected cells, the effect of PGE_2 on tonicity-induced caspase-3 activation was measured. As shown in Fig. 1, caspase-3 activity time-dependently increased after elevating the medium tonicity in the absence of PGE_2. However, when PGE_2 was present from the beginning of the tonicity increase, caspase-3 activity remained at levels not different from isotonic controls, indicating protection from tonicity-induced apoptosis by PGE_2. The same effect was evident when markers of cell damage were assessed. As shown in Fig. 2A, the vast majority of cells detached 20 h after the tonicity increase in the absence of PGE_2. In the presence of PGE_2, however, the cell monolayer remained largely intact (Fig. 2C). Accordingly, PGE_2 significantly prevented LDH release into the medium after elevating the medium osmolality (Fig. 2B).

Effect of PGE_2 on expression of osmoprotective genes

To determine whether protection against tonicity-induced cell damage by PGE_2 is associated with enhanced expression of osmoprotective genes, mRNA abundance of several osmosensitive genes was determined. As demonstrated in Fig. 3, SMIT1, AR and BGT1 mRNA abundance increased time dependently in MDCK cells after raising the medium osmolality. Up-regulation of SMIT and BGT1 preceded that of AR. Addition of PGE_2 further enhanced SMIT and AR mRNA expression, but interestingly, not that of BGT1.

In addition to genes involved in osmolyte accumulation, two other genes with osmoprotective functions, HSP70 and COX-2, were also assessed. As shown in Fig. 3, tonicity-induced up-regulation of HSP70 mRNA was more transient and declined with increased expression of AR, BGT1 and SMIT. In the presence of PGE_2, induction of HSP70 was significantly enhanced. Interestingly, tonicity-induced COX-2 mRNA abundance was further enhanced, suggesting the presence of a positive feedback loop.

Effect of PGE_2 on COX activity

As shown in Fig. 3, induction of COX-2 mRNA in response to osmotic stress was further enhanced in the presence of PGE_2, suggesting the presence of a positive feedback loop. This phenomenon was also evident at the protein level (not shown). To assess whether these effects on COX-2 abundance are accompanied by parallel changes in COX-2 activity, the latter was determined in lysates from cells exposed to osmotic stress in the presence or absence of 20 µm PGE_2. As shown in Fig. 4A, hypertonicity significantly increased COX activity, which was further stimulated about threefold by PGE_2. Addition of DuP-697 (300 nM), a COX-2 specific inhibitor, to the

reaction reduced total COX activity by about 50%, whereas inclusion of SC-560 (300 nM), a COX-1 specific inhibitor, had only minor effects on COX activity, indicating that COX-2 accounts for the vast majority of COX activity and that COX-2 is indeed positively regulated by PGE_2 in response to hypertonicity at the functional level.

Effect of PGE_2 on TonEBP / NFAT5 transcriptional activity

Following osmotic stress, expression of AR, BGT1, SMIT and HSP70 is stimulated by a common transcriptional activator, TonEBP / NFAT5. We therefore determined in the next step, whether the enhanced expression of AR, SMIT and HSP70 in the presence of PGE_2 is related to increased TonEBP / NFAT5 activity.

Enhanced expression and translocation of TonEBP / NFAT5 from the cytosol to the nucleus represents an important mechanism regulating TonEBP / NFAT5 transcriptional activity (Cha et al. 2001). Therefore, nuclear and cytosolic fractions were prepared at different times after the tonicity increase in the presence or absence of PGE_2. As demonstrated in Fig. 5, TonEBP / NFAT5 accumulated rapidly in the nucleus following osmotic stress; however, this translocation was not affected by PGE_2. In cytosolic fractions, TonEBP / NFAT5 abundance was also not different. Likewise, total TonEBP / NFAT5 expression in whole cell lysates was equal in PGE_2 and vehicle-treated cells (not shown). We can, however, not rule out the possibility that changes in TonEBP / NFAT5 transactivation activity, an important mechanism regulating TonEBP / NFAT5 activity (Ferraris et al. 2002), are affected by PGE_2, because this parameter was not assessed in the present study.

TonEBP / NFAT5 reporter activity was then assessed in MDCK cells stably transfected with a TonE-SEAP construct following osmotic stress in the presence and absence of PGE_2. As demonstrated in Fig. 6, TonE-driven SEAP expression time

dependently increased after raising the medium osmolality. This response was, however, not further enhanced by PGE_2. To assess the effect of PGE_2 in the context of a native promoter, additional reporter gene assays were performed using a 1.5 kb fragment of the AR promoter (Fig. 7). In contrast to the results obtained with the TonE-driven construct, PGE_2 significantly stimulated AR promoter-driven reporter activity in response to an increase in tonicity. In aggregate, these data indicate that the presence of TonEs is not sufficient to explain the effect of PGE_2 on AR promoter activity and on the expression of osmoprotective genes. Putative, yet unidentified, factors probably contribute.

Effect of PGE_2 on intracellular electrolytes

In cells exposed to osmotic stress, the intracellular concentration of monovalent ions is increased substantially in the initial phase. Subsequently, intracellular electrolytes are continuously replaced by metabolically neutral organic osmolytes, a process requiring up to several days. To assess whether PGE_2 affects cellular ionic strength (which is determined largely by the sum of intracellular Na^+, Cl^- and K^+ concentrations), MDCK cells were exposed to a gradual tonicity increase to 700 mosmol (kg $H_2O)^{-1}$ over 4 h. After an additional 6 h at 700 mosmol (kg $H_2O)^{-1}$, intracellular electrolytes were determined by electron microprobe analysis. Since progressive cell detachment occurs after 6 h at 700 mosmol (kg $H_2O)^{-1}$ in the absence of PGE_2, extension of the observation period was not feasible. As shown in Fig. 8, cellular ionic strength was elevated substantially after 6 h in MDCK cells exposed to a gradual tonicity increase to 700 mosmol (kg $H_2O)^{-1}$, both in the presence of PGE_2 or vehicle as compared with cells kept in isotonic medium (300 mosmol (kg $H_2O)^{-1}$). Although intracellular concentrations of Na^+ and Cl^- were

less in the presence of PGE_2, those of K^+ were higher than in vehicle-treated cells, thus resulting in comparable cellular ionic strength. The results suggest that organic osmolytes have not been accumulated in significant amounts after 6 h, and that enhanced expression of HSP70 may be more relevant for cytoprotection by PGE_2.

Discussion

Analgesic drug-induced nephropathy accounts for a significant proportion of patients with chronic renal failure (De Broe & Elseviers, 1998). Even short-term use of these compounds may result in impaired renal function, in particular when accompanied by dehydration (Schlöndorff, 1993; De Broe & Elseviers, 1998; Kovacevic et al. 2003). This phenomenon has been traditionally ascribed to an imbalance between medullary vasoconstrictors (i.e. angiotensin II, arginine–vasopression, endothelin and others) to medullary vasodilators (i.e. nitric oxide, prostacyclin, PGE_2), thus resulting in hypoxic damage of medullary cells (Schlöndorff, 1993; Brezis & Rosen, 1995). In particular, the production of the latter two substances is impeded by NSAIDs, indicating that prostanoids may contribute decisively to the survival of medullary cells in conditions associated with stimulation of the urinary concentrating mechanism.

During antidiuresis, the renal inner medulla is not only chronically hypoxic, but is also characterized by extreme interstitial solute concentrations (Neuhofer & Beck, 2005). It is thus conceivable that medullary prostanoids not only improve medullary oxygen balance but also favour the adaptation of medullary cells to high interstitial solute concentrations. In agreement, PGE_2, the major medullary prostanoid, is produced in large amounts in the renal medulla during antidiuresis (Breyer & Breyer, 2001; Yang, 2003).

Indeed, the present observations clearly demonstrate that PGE_2 promotes survival of MDCK cells exposed to osmotic stress, which coincided with enhanced expression of osmoprotective genes (SMIT, AR, HSP70). In the present experiments, extracellular tonicity was raised almost linearly over several hours, an approach which more closely resembles the situation in the renal papilla *in situ*, than does a one-step

elevation that has been employed in most experimental models to date. A comparable increase in interstitial tonicity has been reported in the papilla of the mammalian kidney *in vivo* following acute stimulation of the urinary concentrating mechanism (Atherton et al. 1971). Enhanced cell survival has been directly demonstrated following a gradual increase in osmolality (Cai et al. 2002). In contrast to a step increase, cells exposed to a gradual tonicity increase are unlikely to show a significant reduction in cell volume by virtue of the concurrent operation of volume regulatory mechanisms, a situation known as isovolumetric regulation (Lohr & Grantham, 1986). This is achieved by gradual uptake of electrolytes from the extracellular space and intracellular accumulation of organic osmolytes with increasing extracellular osmolality and osmotically obliged water entry, thus allowing maintenance of cell volume (Rome et al. 1989). Nonetheless, both a step and a gradual tonicity increase are associated with a rise in cellular ionic strength, which exerts adverse effects on cell viability, including DNA double strand breaks and induction of apoptosis (Galloway et al. 1987; Kültz & Chakravarty, 2001). In the longer term, renal medullary cells both *in vivo* and *in vitro* adapt to elevated extracellular solute concentrations by the accumulation of osmotically active, non-perturbing organic osmolytes, which replace intracellular ions. In conjunction with osmotic changes, there is enhanced expression of cytoprotective heat shock proteins, mainly HSP70 (Cohen *et al.* 1991; Neuhofer & Beck, 2005).

The accumulation of significant amounts of organic osmolytes is, however, a relatively slow process, requiring up to several days. In agreement, in the present experiments, cellular ionic strength 6 h after a gradual tonicity increase was elevated to comparable levels in the presence of PGE_2 or vehicle. These observations argue against the accumulation of significant amounts of organic osmolytes in this early

phase. Nevertheless, caspase-3 activation at this time is completely prevented by PGE_2 and cell survival is strongly enhanced after 20 h (Figs 1 and 2). As shown in Fig. 3, induction of HSP70 precedes that of SMIT and AR, suggesting that HSP70 may protect medullary cells in a situation in which compatible osmolytes are not yet present in sufficient amounts. In agreement, HSP70 potently blocks apoptosis at several levels, including direct interaction with caspase-3, thus impairing the execution of the apoptotic pathway (Ravagnan et al. 2001; Komarova et al. 2004). In addition to the increased mRNA abundance of HSP70 in the presence of PGE_2, HSP70 was also induced more vigorously at the protein level when the hypertonic medium was supplemented with PGE_2 (not shown). Thus, in the early phase of osmotic stress, HSP70 may be important for cell survival.

Tonicity-stimulated expression of AR, BGT1, SMIT and HSP70 has been demonstrated to be mediated by TonEBP / NFAT5 (Woo et al. 2002 a, b). Although the activity of TonEBP / NFAT5 is required for tonicity-induced expression of osmoprotective genes (Na et al. 2003; Lopez-Rodriguez et al. 2004), the present data demonstrate enhanced expression of AR, SMIT, HSP70 and COX-2 in the presence of PGE_2 in the absence of increased nuclear abundance or elevated TonE-driven reporter activity. Nevertheless, PGE_2 significantly increased reporter activity in experiments using a 1.5 kb fragment of the AR promoter. These data indicate that the presence of TonEs is not sufficient to explain the effect of PGE_2 on AR promoter activity and expression of osmoprotective genes. Probably, additional signalling events and transcriptional activators modulate the expression of osmoprotective genes, possibly via cooperative effects on TonEBP. This assumption is supported further by the lack of stimulation of BGT1 expression. Divergent pathways regulating the expression of SMIT and BGT1 have already been previously described (Atta et

al. 1999). Additionally, tonicity-induced expression of COX-2 was also markedly stimulated by PGE_2, although COX-2 is not a known TonEBP / NFAT5 target gene (Yang, 2003), further supporting the view that the stimulatory effect of PGE_2 is not directly mediated via enhanced TonEBP-TonE signalling.

In summary, PGE_2 promotes survival of MDCK cells exposed to osmotic stress, which occurred in parallel with enhanced expression of osmoprotective genes. The latter effect appears, however, not to be directly mediated by increased transcriptional activity of TonEBP / NFAT5. Tonicity-induced expression of COX-2 and activity was further stimulated by PGE_2, indicating the existence of a positive feedback loop. This observation may reflect the requirement for the rapid production of PGE_2 after stimulation of the urinary concentrating mechanism. Thus, elevated PGE_2 concentrations during antidiuresis may not only help to prevent hypoxic injury to medullary cells, but also to promote osmoadaptation. The present concept that PGE_2 promotes osmoadaptation of medullary cells is in good agreement with the observation that COX inhibition may be associated with renal papillary injury *in vivo* (Moeckel et al. 2003; Neuhofer et al. 2004), particularly when accompanied by dehydration.

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Legends for figures

Figure 1. Effect of prostaglandin E_2 (PGE_2) on tonicity-induced caspase-3 activation.
MDCK cells were exposed to a gradual tonicity increase to 700 mosmol (kg H_2O)$^{-1}$ over 4 h by NaCl addition in the presence of 20 µM PGE_2 (*NaCl* + *PGE_2*) or vehicle ethanol (*NaCl* + *vehicle*). At the times indicated after reaching the final osmolality of 700 mosmol (kg H_2O)$^{-1}$, relative caspase-3 activity was determined as described in the Methods. Means±SEM for *n*=4-6; **P*<0.05 vs. *NaCl* + *PGE_2*.

Figure 2. Effect of PGE_2 on cell survival of MDCK cells under hypertonic conditions.
MDCK cells were exposed to a gradual tonicity increase to 700 mosmol (kg H_2O)$^{-1}$ over 4 h by NaCl addition in the presence of 20 µM PGE_2 (*NaCl* + *PGE_2*) or vehicle ethanol (*NaCl* + *vehicle*). Twenty hours after reaching the final osmolality of 700 mosmol (kg H_2O)$^{-1}$, cell survival was assessed. (A) Percentage of surviving cells determined as described in Methods. Means±SEM for *n*=4; * *P*<0.05 vs. *NaCl* + *vehicle*. (B) Lactate dehydrogenase (LDH) release expressed as percentage of total LDH activity of the respective cultures. Means±SEM for *n*=4; * *P*<0.05 vs. *NaCl* + *vehicle*. (C) Representative phase contrast micrographs of the cells remaining attached to the culture dish 20 h after tonicity increase.

Figure 3. Effect of PGE_2 on expression of osmoprotective genes in MDCK cells under hypertonic conditions.
MDCK cells were exposed to a gradual tonicity increase to 500 mosmol (kg H_2O)$^{-1}$ by NaCl addition in the presence of 20 µM PGE2 (*NaCl* + *PGE_2*) or vehicle ethanol (*NaCl* + *vehicle*), or were kept in isotonic medium (*iso*). The expression of the

respective genes (SMIT, sodium *myo*-inositol cotransporter-1; AR, aldose reductase; BGT1, betaine/GABA transporter-1; HSP70, heat shock protein 70; COX-2, cyclooxygenase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase) was determined at the times indicated after reaching the final osmolality of 500 mosmol (kg H_2O)$^{-1}$ by Northern blot analysis. (A) Relative mRNA abundance was normalized to that of GAPDH. Means±SEM for *n*=4; **P*<0.05 vs. *NaCl + vehicle*. (B) Representative Northern blots.

Figure 4. Effect of PGE_2 on COX activity in MDCK cells exposed to osmotic stress.
MDCK cells were exposed to a gradual tonicity increase to 500 mosmol (kg H_2O)$^{-1}$ by NaCl addition in the presence of 20 µM PGE_2 (*NaCl + PGE_2*) or vehicle ethanol (*NaCl + vehicle*), or were kept in isotonic medium (*iso*). (A) After 24 h, COX activity was determined as described in "Methods". Means±SEM for *n*=4; **P*<0.05 vs. *iso*; #*P*<0.05 vs. *NaCl + Vehicle*. (B) The contribution of COX-1 and COX-2 isoforms to total COX activity in lysates from cells treated with NaCl in the presence of PGE_2 was assessed by addition of COX-1 (*SC-560*) and COX-2 (*Du-P697*) specific inhibitors, respectively. Means±SEM for *n*=4.

Figure 5. Effect of PGE_2 on nuclear and cytosolic abundance of tonicity-responsive enhancer binding protein/nuclear factor of activated T cells-5 (TonEBP / NFAT5) under hypertonic conditions.
MDCK cells were exposed to a gradual tonicity increase to 500 mosmol (kg H_2O)$^{-1}$ by NaCl addition in the presence of 20 µM PGE_2 (*NaCl + PGE_2*) or vehicle ethanol (*NaCl + vehicle*), or were kept in isotonic medium (*iso*). At the times indicated after reaching the final osmolality of 500 mosmol (kg H_2O)$^{-1}$, nuclear and cytosolic

fractions were prepared and relative TonEBP / NFAT5 abundance within each fraction was determined by Western blot analysis. Means±SEM for *n*=3-4.

Figure 6. Effect of PGE_2 on TonE-driven reporter activity.

MDCK cells were stably transfected with a TonE-driven secreted alkaline phosphatase (SEAP) reporter construct. Subsequently, the cells were exposed to a gradual tonicity increase to 500 mosmol (kg $H_2O)^{-1}$ by NaCl addition in the presence of 20 µM PGE_2 (*NaCl + PGE_2*) or vehicle ethanol (*NaCl + vehicle*), or were kept in isotonic medium (*iso*). Relative SEAP activity in the cell culture supernatant was assessed at the indicated time points as described in Methods. Means±SEM for *n*=4-8; **P*<0.05 vs. *iso.*

Figure 7. Effect of PGE_2 on aldose reductase promoter activity.

MDCK cells were transiently transfected with a 1,5 kb aldose reductase promoter-driven secreted alkaline phosphatase (SEAP) reporter construct. Subsequently, the cells were exposed to a gradual tonicity increase to 500 mosmol (kg $H_2O)^{-1}$ by NaCl addition in the presence of 20 µM PGE_2 (*NaCl + PGE_2*) or vehicle ethanol (*NaCl + vehicle*), or were kept in isotonic medium (*iso*). Relative SEAP activity in the cell culture supernatant was assessed 24 h after tonicity increase as described in "Methods". Means±SEM for *n*=6. **P*<0.05 vs. *iso* ; #*P*<0.05 vs. *NaCl + Vehicle*.

Figure 8. Effect of PGE_2 on intracellular electrolyte concentrations in MDCK under hypertonic conditions.

MDCK cells were exposed to a gradual tonicity increase to 700 mosmol (kg $H_2O)^{-1}$ by NaCl addition in the presence of 20 µM PGE_2 (*NaCl + PGE_2*) or vehicle ethanol

(*NaCl + vehicle*), or kept in isotonic medium (*iso*). Then, 6 h after reaching the final osmolality of 700 mosmol (kg $H_2O)^{-1}$, intracellular concentrations of Na^+, Cl^- and K^+ were determined by electron microprobe analysis. The number of intracellular measurement (*N*) is indicated. **P*<0.05 vs. *iso* ; ‡*P*<0.05 vs. *NaCl + Vehicle* for Na^+ and K^+.

Figures

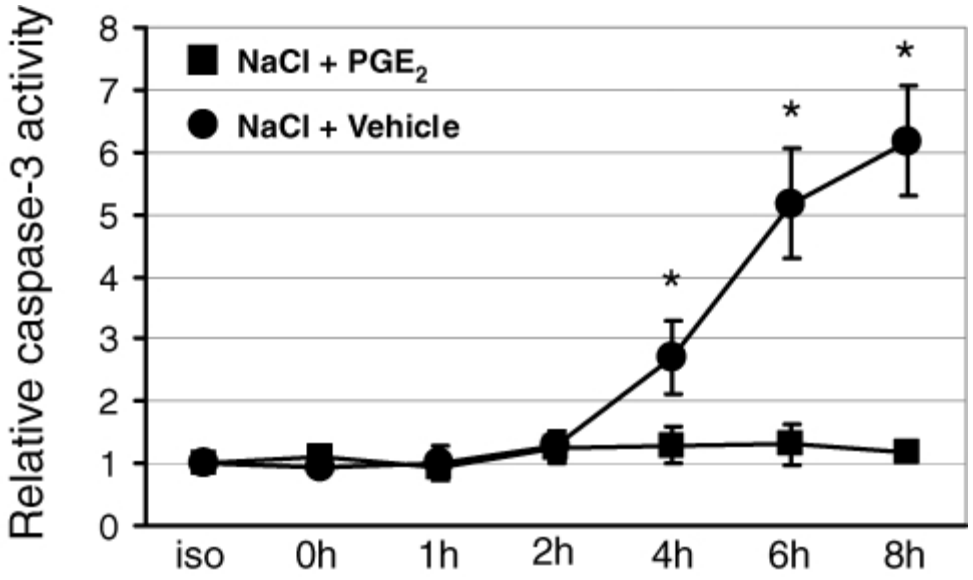
Relative caspase-3 activity
0
1
2
3
4
5
6
7
8
NaCl + PGE2
NaCl + Vehicle
*
*
*
iso
0h
1h
2h
4h
6h
8h

Figure 1

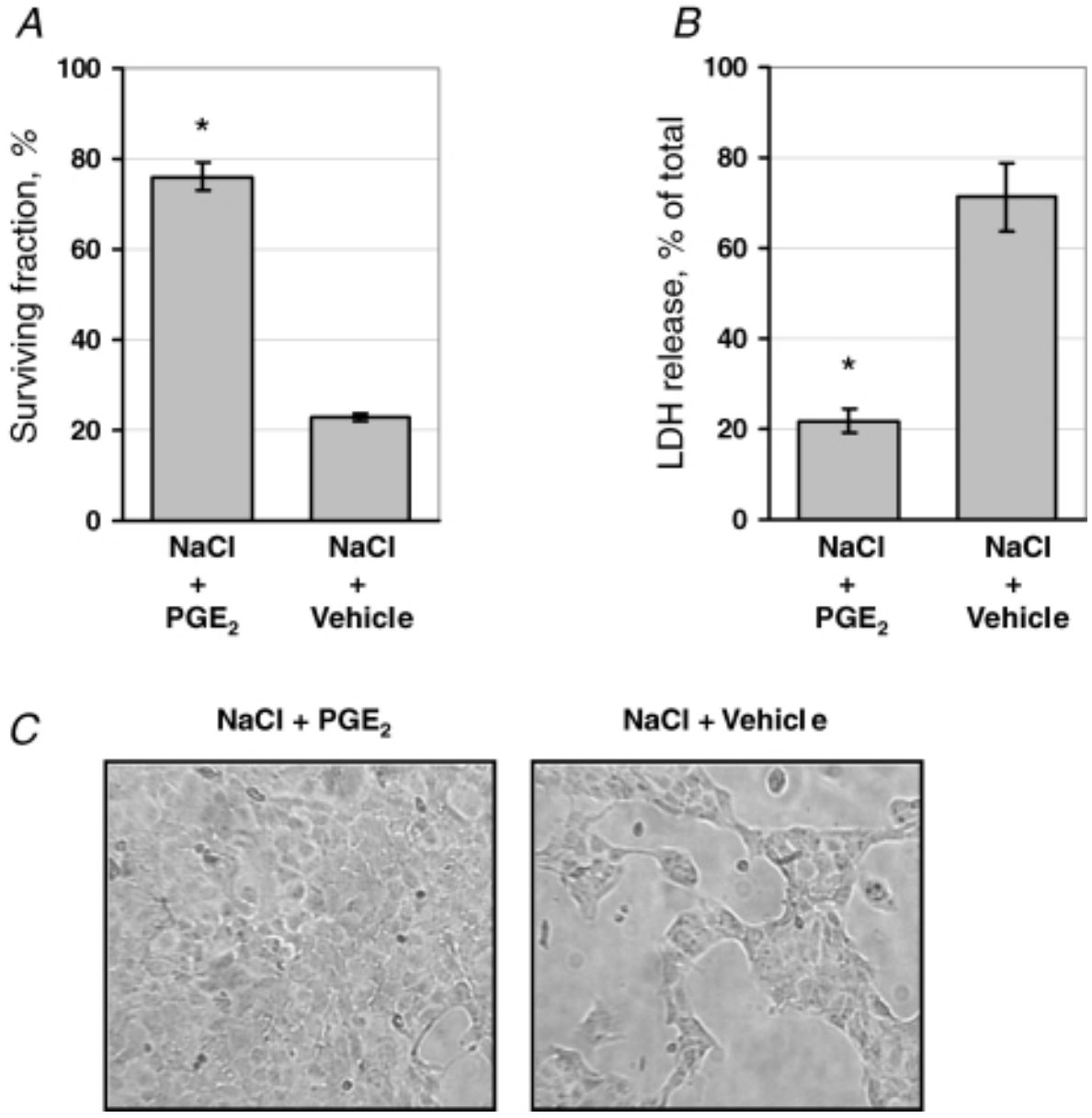
A
Surviving fraction, %
100
80
60
40
20
0
*
NaCl + PGE2
NaCl + Vehicle
B
LDH release, % of total
100
80
60
40
20
0
*
NaCl + PGE2
NaCl + Vehicle
C
NaCl + PGE2
NaCl + Vehicle

Figure 2

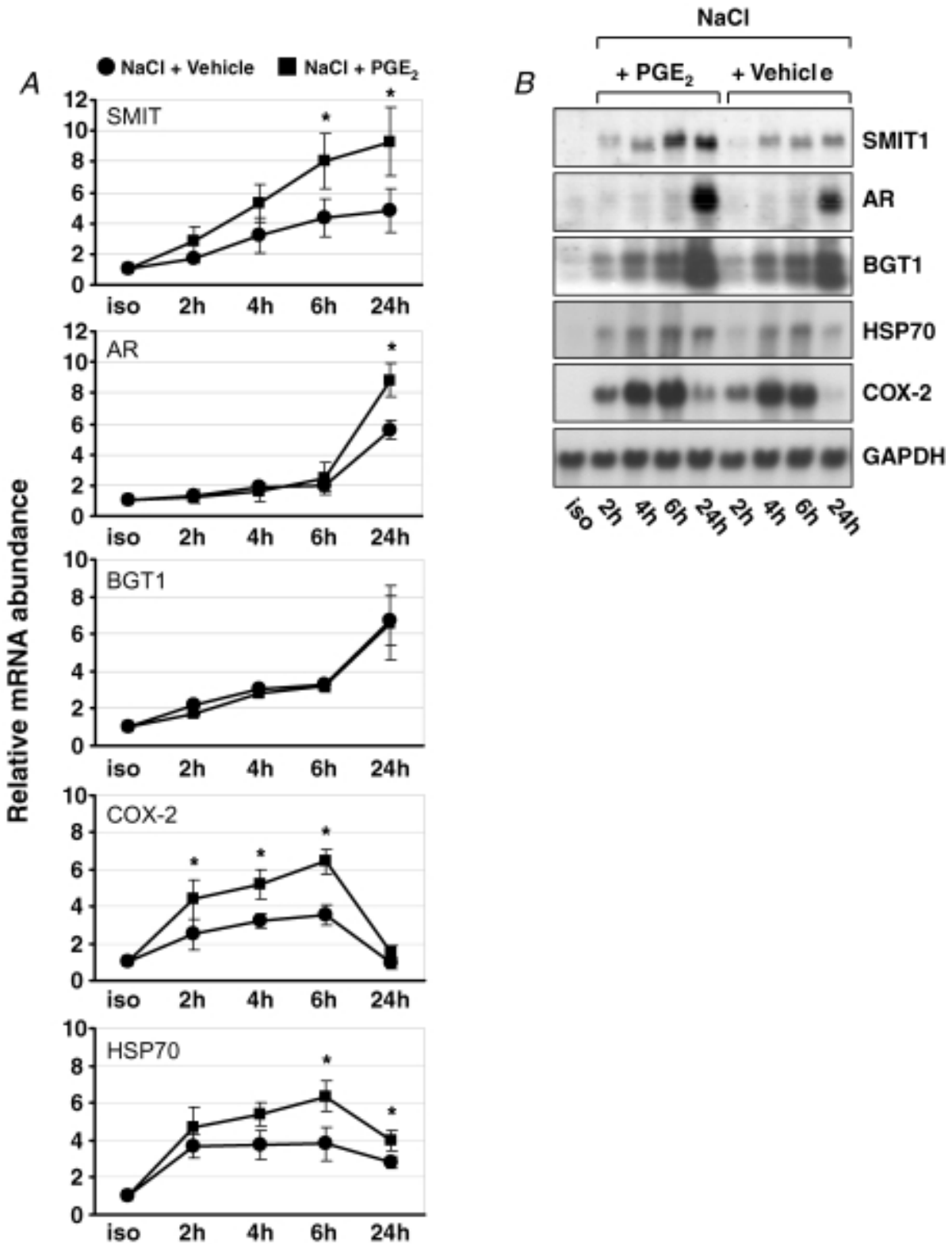
A
NaCl + Vehicle
NaCl + PGE2
SMIT
AR
BGT1
COX-2
HSP70
Relative mRNA abundance
iso
2h
4h
6h
24h
B
NaCl
+ PGE2
+ Vehicle
SMIT1
AR
BGT1
HSP70
COX-2
GAPDH

Figure 3

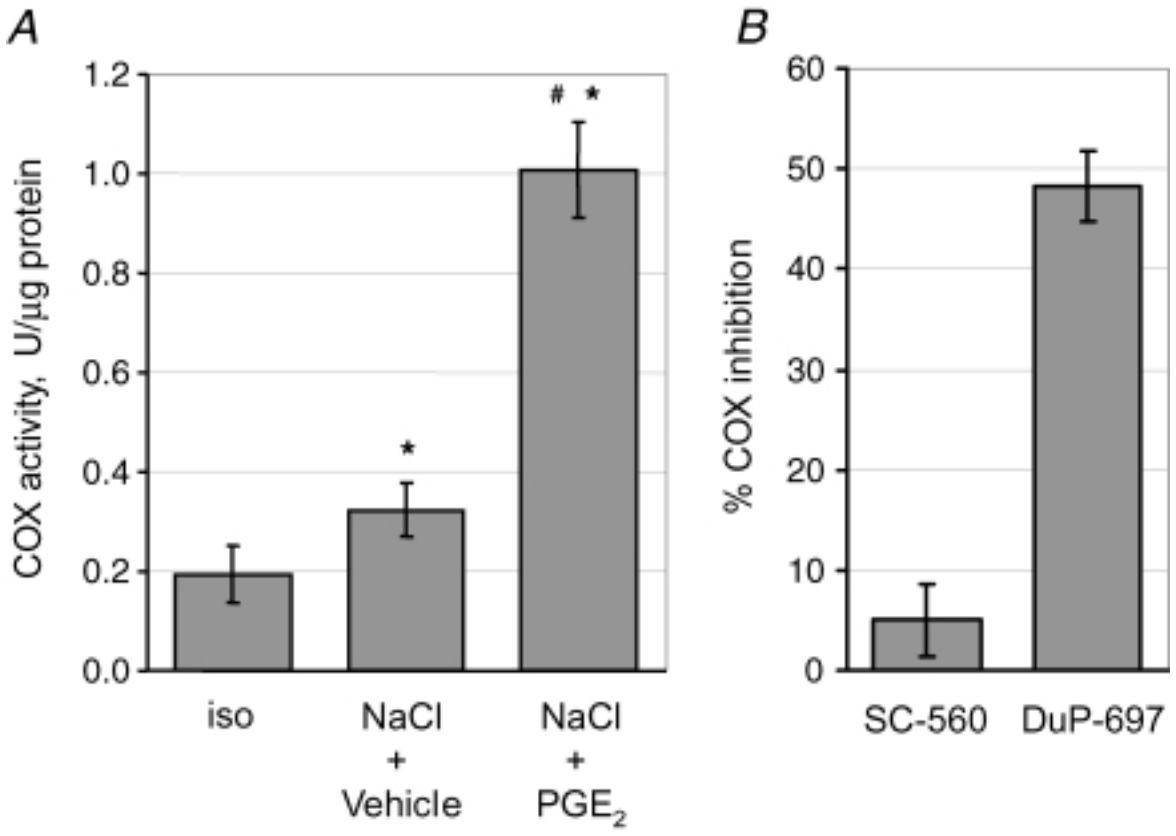
A
COX activity, U/µg protein
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
iso
NaCl + Vehicle
NaCl + PGE2
B
% COX inhibition
60
50
40
30
20
10
0
SC-560
DuP-697

Figure 4

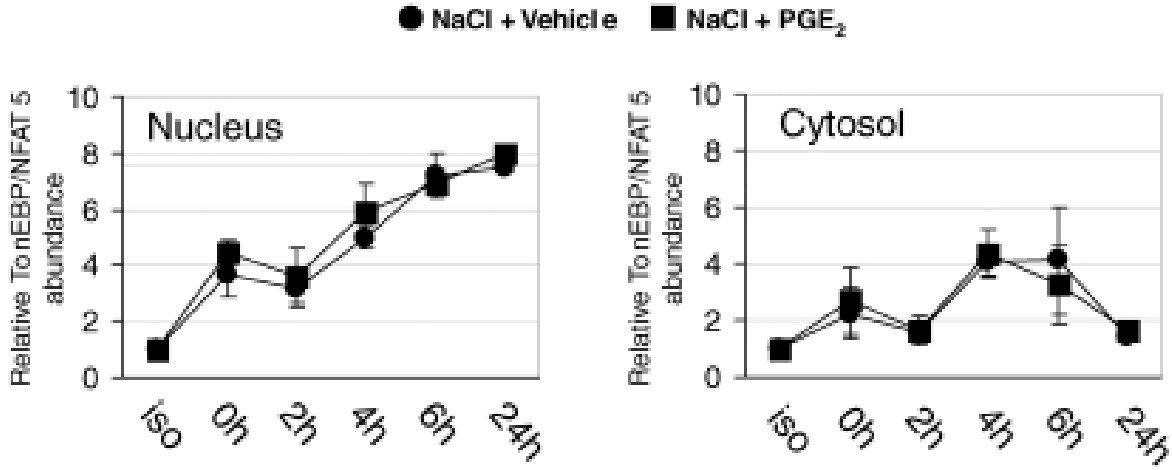
NaCl + Vehicle
NaCl + PGE2
Nucleus
Cytosol
Relative TonEBP/NFAT 5 abundance
0
2
4
6
8
10
iso
0h
2h
4h
6h
24h

Figure 5

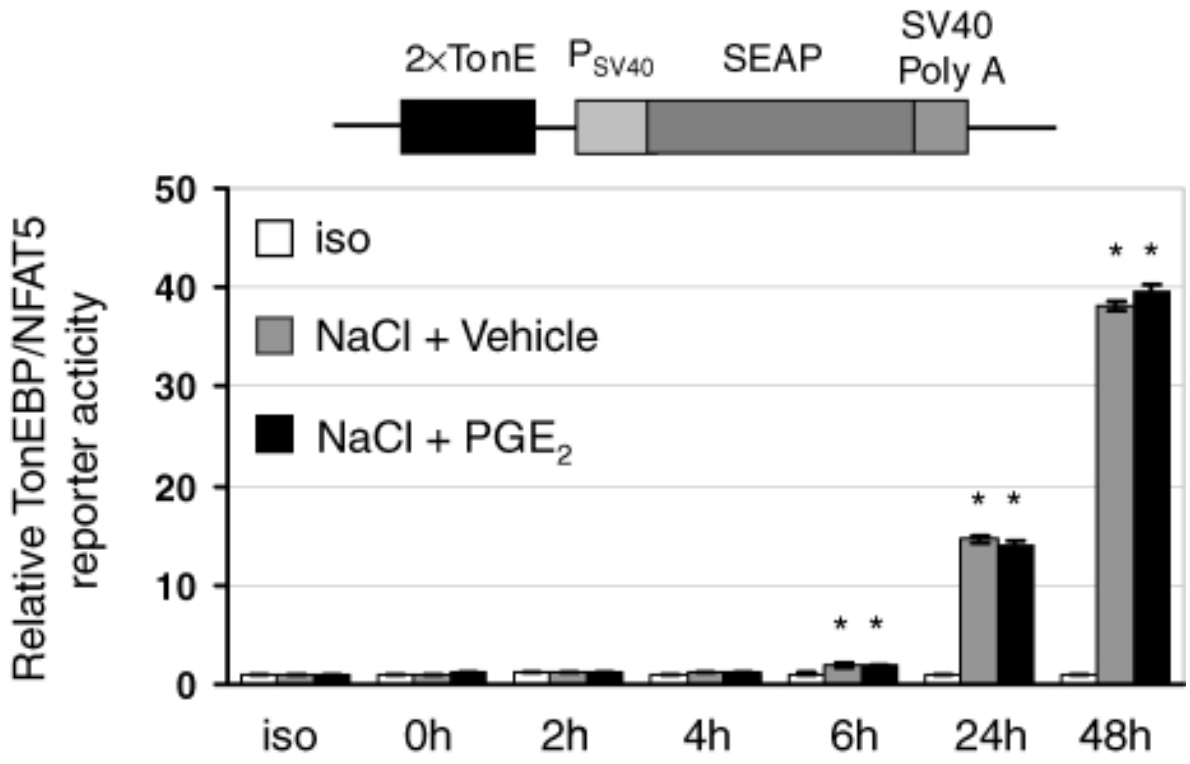
2×TonE
P_{SV40}
SEAP
SV40
Poly A
Relative TonEBP/NFAT5
reporter acticity
50
40
30
20
10
0
iso
NaCl + Vehicle
NaCl + PGE_2
* *
* *
* *
iso
0h
2h
4h
6h
24h
48h

Figure 6

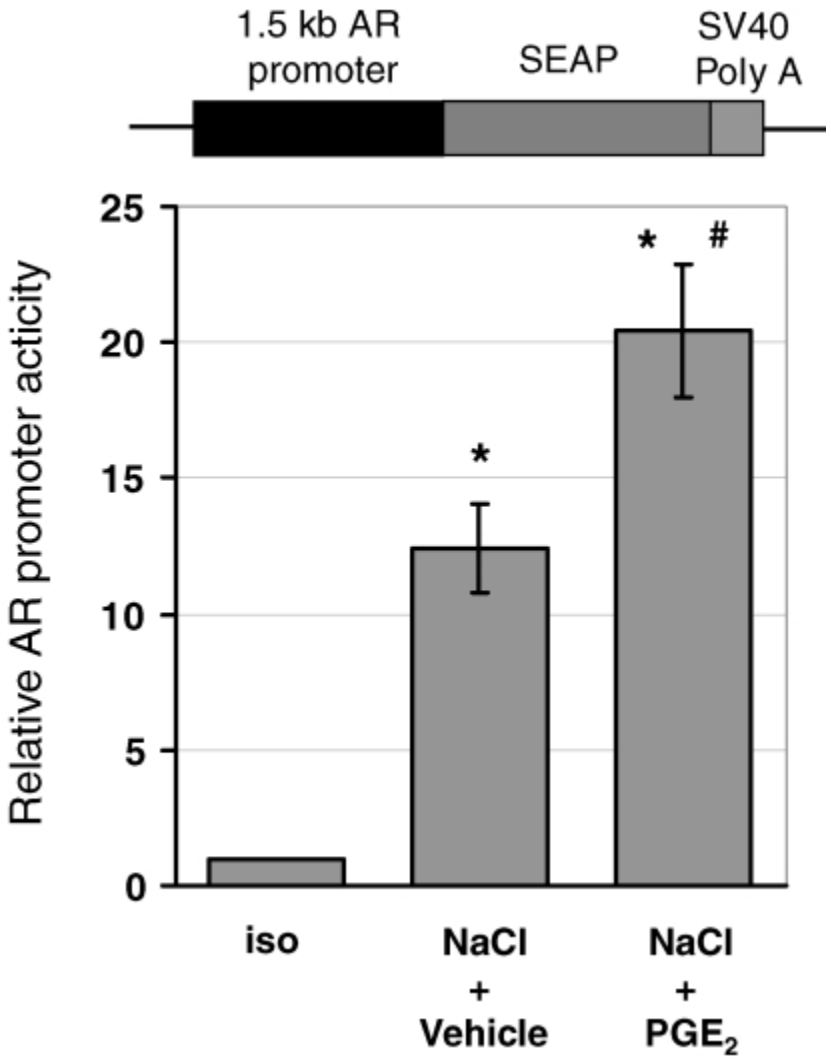
1.5 kb AR promoter
SEAP
SV40 Poly A
Relative AR promoter acticity
25
20
15
10
5
0
*
*
#
iso
NaCl + Vehicle
NaCl + PGE2

Figure 7

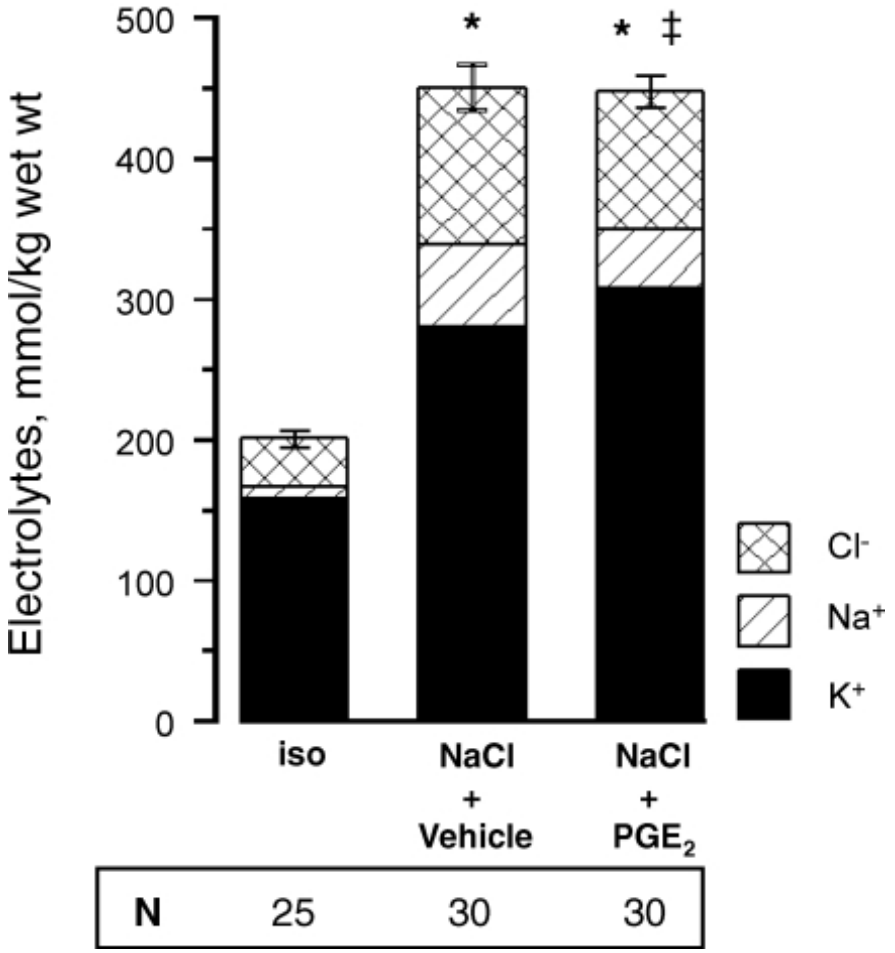
Electrolytes, mmol/kg wet wt
500
400
300
200
100
0
*
* ‡
Cl-
Na+
K+
iso
NaCl + Vehicle
NaCl + PGE2
N
25
30
30

Figure 8

2. 2. PGE_2 potentiates tonicity-induced COX-2 expression in renal medullary cells in a positive feedback loop involving EP2-cAMP-PKA signaling

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PGE_2 potentiates tonicity-induced COX-2 expression in renal medullary cells in a positive feedback loop involving EP2-cAMP-PKA signaling

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Running title: PGE_2 stimulates COX-2 expression

Key words: COX-2, prostaglandin E_2, osmoadaptation, positive feedback, apoptosis, survival factor

Abstract

Cyooxygenase-2 (COX-2)-derived PGE_2 is critical for the integrity and function of renal medullary cells during antidiuresis. The present study extended our previous finding that tonicity-induced COX-2 expression is further stimulated by the major COX-2 product PGE_2 and investigated the underlying signaling pathways and the functional relevance of this phenomenon. Hyperosmolality stimulated COX-2 expression and activity in Madin-Darby canine kidney (MDCK) cells, a response that was further increased by PGE_2-cAMP signaling, suggesting the existence of a positive feedback loop. This effect was diminished by AH-6809, an EP2 antagonist, and by the PKA inhibitor H-89, but not by AH-23848, an EP4 antagonist. The effect of PGE_2 was mimicked by forskolin and dibutyryl-cAMP, suggesting that the stimulatory effect of PGE_2 on COX-2 is mediated by a cAMP-PKA-dependent mechanism. Accordingly, cAMP-responsive element (CRE)-driven reporter activity paralleled the effects of PGE_2, AH-6809, AH-23848, H-89, forskolin, and dibutyryl-cAMP on COX-2 expression. In addition, the stimulatory effect of PGE_2 on tonicity-induced COX-2 expression was blunted in cells transfected with dominant-negative CRE binding (CREB) protein, as was the case in a COX-2 promoter reporter construct in which a putative CRE was deleted. Furthermore, PGE_2 resulted in PKA-dependent phosphorylation of the pro-apoptotic protein Bad at Ser155, a mechanism that is known to inactivate Bad, which coincided with reduced caspase-3 activity during osmotic stress. Conversely, pharmacological interruption of the PGE_2-EP2-cAMP-PKA pathway abolished Ser155 phosphorylation of Bad and blunted the protective effect of PGE_2 on cell survival during osmotic stress. These observations indicate the existence of a positive feedback loop of PGE_2 on COX-2 expression during osmotic

stress, an effect that apparently is mediated by EP2-cAMP-PKA signaling, and that contributes to cell survival under hypertonic conditions.

Introduction

The interstitial environment of the renal medulla is characterized by extremely high interstitial osmolalities and low oxygen tension (34). Nevertheless, medulla-resident cells not only have the ability to survive in this hostile environment but also to function normally. This feature is of integral importance for maintaining the kidneys' function in regulating systemic electrolyte and fluid homeostasis.

A characteristic feature of renal medullary cells is their high capacity for production of prostaglandins (PG) (4). PGs are autacoids acting close to their site of formation and contribute essentially to the regulation of tubular solute and water reabsorption and adjusting medullary blood flow to metabolic requirements (12, 32, 34). Moreover, PGs promote the adaptation of renal medullary cells to high extracellular osmolalities by virtue of enhancing the expression of osmoprotective genes (29, 35, 37). The formation of PGs depends on the sequential action of cytosolic phospholipase A_2 ($cPLA_2$), which releases arachidonic acid from membrane phospholipids, and cyclooxygenase (COX) (30). The direct metabolite of arachidonic acid formed by COX is PGH_2, which is then converted to various biologically active prostanoids. At least two different COX isoforms have been described; these share the same enzymatic properties but differ substantially in their amino acid sequence and mode of regulation. In contrast to most other tissues, COX-2 is expressed constitutively in the renal medulla, is further upregulated during dehydration, and is assumed to account for the majority of prostanoids formed in the renal medulla under this condition (7, 49). Among the various prostanoids, PGE_2 accounts quantitatively for the vast majority of PGs formed in the renal medulla (4). Four PGE_2 receptor subtypes, designated EP1 to EP4, mediate the biological effects of PGE_2. Activation

of EP1 causes mobilization of Ca^{2+}, while EP3 is coupled to $G_{\alpha i}$, thereby inhibiting intracellular cAMP formation (31). Both EP2 and EP4 are linked to $G_{\alpha s}$, which results in enhanced cAMP levels upon receptor stimulation (31).

In the renal papilla in vivo, COX-2 is robustly induced by arginine vasopressin (AVP) administration as well as by dehydration, and COX-2 abundance closely correlates with urine osmolality (49, 51). Further studies have demonstrated that the enhanced expression of COX-2 during antidiuresis is not mediated directly by AVP but occurs secondarily in response to a rise in medullary interstitial tonicity (49, 51). In contrast to other osmosensitive genes, COX-2 is not regulated by tonicity-responsive enhancer binding protein/nuclear factor of activated T cells 5 (TonEBP/NFAT5), which drives the expression of several genes required for both efficient urinary concentration and osmoadaptation of renal medullary cells (46). The importance of enhanced COX-2 expression and PGE_2 production under hypertonic conditions is underscored by the fact that COX-2 induction precedes that of other osmoprotective genes during osmotic stress (37). Consistent with this notion, diminished COX-2 expression or selective inhibition of COX-2 is associated with damage of renal medullary cells in vivo and in vitro following a rise in ambient tonicity (13, 29, 35). Thus the rapid induction of COX-2 and subsequent production of PGE_2 appears to be of critical importance for efficient osmoadaptation of renal medullary cells. In the renal papilla in vivo, interstitial tonicity may increase two- to threefold within 2–4 h after acute stimulation of the urinary concentrating mechanism (1). Comparable increases in osmolality, however, cause apoptosis in cultured medullary cells, while PGE_2 confers protection when added to the medium (25, 37). PGE_2 has been demonstrated to exert anti-apoptotic effects, which are mediated by EP2 and EP4 receptors, in various cell types (2, 6, 42).

We have previously observed that PGE_2 enhances the expression of various osmoprotective genes and, interestingly, also that of COX-2 under hypertonic conditions, suggesting the existence of a positive feedback loop. This is consistent with a previous study by Sonoshita et al. (41) who demonstrated stimulation of COX-2 expression in an EP2 receptor, PKA- dependent manner in a murine intestinal polyposis model (41). It thus appears possible that PGE_2 potentiates the induction of COX-2 during osmotic stress in an auto-/paracrine manner, thereby further increasing PGE_2 production in a positive feedback loop. This mechanism would, in turn, favor cell survival by antagonizing pro-apoptotic signaling pathways that are activated during acute osmotic stress. The aim of the present study was thus to extend our previous findings with focus on the signaling pathways and to address the functional significance in Madin-Darby canine kidney (MDCK) cells as a model system for renal medullary cells.

Methods

Cell culture and experimental protocol.

MDCK cells were obtained from the American Type Culture Collection (ATCC; CCL 34). The cells were grown under standard conditions in Dulbecco's Modified Eagles Medium (DMEM) (low glucose) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. For experiments, the cells were plated in 80-mm, 35-mm (Greiner, Frickenhausen, Germany), or 24-well plates (Nunc, Roskilde, Denmark). After confluence was reached, medium tonicity was increased to the indicated final osmolality by adding the required volume of 4 M NaCl drop by drop to the dishes. To achieve a quasilinear, gradual increase in the medium tonicity, the latter was elevated in 10 steps of 40 mosmol/kg H_2O each over a period of 4 h to a final osmolality of 700 mosmol/kg H_2O. In experiments with pharmacological inhibitors, the respective compound was added at the indicated concentration from concentrated stock solutions 30 min before tonicity increase, or, as a control, the same volume of vehicle was added. Stock solutions were prepared as following: AH6809 (10 mM in DMSO; Sigma, Schnelldorf, Germany), AH23848 (30 mM in DMSO; Sigma), and H-89 (20 mM in DMSO; Sigma). In experiments with PGE_2 (10 mM stock in ethanol; Sigma), forskolin (1 mM stock in ethanol; Sigma), or dibutyryl-cAMP (1 M stock in H_2O; Roche Diagnostics, Indianapolis, IN), the respective agonist or the same volume of vehicle was added at the time of tonicity increase.

Western blot analysis.

For detection of COX-2 and actin, cells were washed with ice-cold PBS and lysed in 8 M urea-PBS (75 µl / 35-mm plate) and centrifuged at 13,000 g for 15 min at 4°C.

For immunodetection of Bad and phospho-Bad-Ser155, the cells in 35-mm dishes were washed with ice-cold Tris-buffered saline (TBS), incubated for 5 min on ice in 50 µl lysis buffer containing (in mM) 20 Tris·HCl, pH 7.5, 150 NaCl, 1 EDTA, 1 EGTA, 1% (vol/vol) Triton X-100, 1% (vol/vol) broad spectrum protease inhibitor cocktail (Sigma), and 1% (vol/vol) phosphatase inhibitor cocktail 2 (Sigma). Thereafter, the cells were collected with a rubber policeman, sonicated four times for 5 s on ice, and centrifuged at 13000 g for 10 min at 4°C.

Protein concentration in the supernatants was determined using a commercially available assay (Bio-Rad, Hercules, CA) and aliquots of 20 µg (COX-2, actin) or aliquots of 80 µg (Bad, phospho-Bad) of protein were separated on 10% (COX-2, actin) or 12% (Bad, phospho-Bad) SDS gels and subsequently transferred to nitrocellulose membranes (Hybond; Amersham, Freiburg, Germany). For COX-2 and actin staining, nonspecific binding sites were blocked by incubation in blocking buffer (5% nonfat dry milk in PBS containing 0.1% Tween-20). COX-2 abundance was determined by incubation with rabbit polyclonal anti-COX-2 antibody (1:5000 in blocking buffer; Cayman Chemicals, Ann Arbor, MI) at 4°C overnight. Actin was stained by incubation with rabbit polyclonal anti-actin antibody (1:5,000 in blocking buffer; 1 h at room temperature; Sigma). Expression of EP2 and cAMP responsive element (CRE) binding (CREB) was monitored by rabbit polyclonal anti-EP2 (1:1,000 in blocking buffer; Santa Cruz Biotechnology;) and by rabbit polyclonal anti-CREB (1:1,000 in blocking buffer; Cell Signaling Technology). Subsequently, the membranes were incubated for 1 h with horseradish peroxidase -conjugated goat anti-rabbit antibody (1:5,000; Jackson Immuno-Research, West Grove, PA) at room temperature. Finally, immunocomplexes were detected by enhanced chemiluminescence (Pierce, Rockford, IL) and signals quantified using Image J

software (NIH, Bethesda, MD). For staining of Bad and phospho-Bad, the membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween-20 at room temperature for 1 h, washed 3x for 5 min in TBS-0.1% Tween-20, and incubated with the respective antibody in 5% BSA in TBS-0.1% Tween-20 at 4°C overnight. Total Bad protein and Bad phosphorylated at Ser155 were detected using polyclonal rabbit anti-Bad antibody (1:1,000; Cell Signaling Technology) and polyclonal anti phospho-Bad-Ser155 antibody (1:1,000; Cell Signaling Technology), respectively. Incubation with secondary antibody and detection procedures were performed as described above.

Northern blot analysis.

Cells were lysed by adding TriFast reagent (1 ml/85 mm dish; PeqLab, Erlangen, Germany), and total RNA was isolated according to the manufacturer's recommendations. Aliquots (20 µg) were separated on 1.0% agarose-formaldehyde gels, transferred to nylon membranes (Roche, Mannheim, Germany), and immobilized by UV crosslinking. Relative abundance of the respective mRNAs was monitored by hybridization with biotin-labeled cDNA probes specific for COX-2 and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as described in detail previously (37). After hybridization, the membranes were washed twice with 2 x SSC-0.1% SDS at room temperature and twice with 1 x SSC in 0.1% SDS at 50°C. Nonradioactive detection procedures were performed using a commercially available kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Band intensity was quantified using Image J software, and COX-2 mRNA abundance was normalized to that of GAPDH to correct for differences in RNA loading.

Reporter gene assays.

To monitor activation of potential signaling pathways, MDCK cells were transfected stably with appropriate reporter constructs based on secreted alkaline phosphatase (SEAP; Clontech, Palo Alto, CA). (1) In pCRE-SEAP (Clontech), expression of SEAP is under control of a CRE; (2) In pNF-κB-SEAP (Clontech), transcription of SEAP is driven by a nuclear factor (NF)-κB site (3); pTonE-SEAP is controlled by a tandem tonicity-responsive element (TonE) motive and was constructed as described previously (37). MDCK cells in 85-mm dishes were transfected with 20 µg DNA at a 20:1 molar ratio of the respective SEAP construct and pcDNA3-LacZ (Invitrogen, Karlsrühe, Germany) as described in detail elsewhere (37). After the selection with 600 µg/ml geneticine (Sigma), 24 clones for each construct were expanded and assayed for inducible reporter gene expression after stimulation with 20 µM forskolin (pCRE-SEAP), 10 ng / ml tumor necrosis factor-α (pNF-κB-SEAP), or by adding an additional 100 mM NaCl to the medium (pTonE-SEAP). Clones with highly inducible SEAP expression were pooled and used in experiments at passages 3–10.

In reporter assays, the cells were seeded in 24-well plates. After reaching confluence, the medium containing geneticine was replaced by DMEM without phenol red lacking geneticine, and the cells were treated as indicated. After 24 h, 50 µl medium was transferred to a 96-well plate that was sealed and incubated in a water bath at 65°C for 30 min to inactivate endogenous alkaline phosphatase activity. For SEAP detection, 150 µl p-nitrophenyl phosphate liquid substrate (Sigma) was added, and samples were incubated at room temperature for appropriate periods. Finally, SEAP activity was assessed at 405 nm in a microplate reader.

Generation of MDCK cells expressing dominant-negative CREB.

For disruption of intracellular cAMP-PKA signaling, MDCK cells were transfected with the plasmid pCMV-KCREB (Clontech), encoding a mutant variant of the human CREB protein that contains mutations in the DNA-binding domain, which forms dimers with endogenous CREB and blocks the ability of endogenous CREB to bind to CRE (45). Transfections were performed using the calcium phosphate method as previously described (36). After selection of stable clones with 800 µg / ml geneticine, 24 clones obtained for each vector construct were expanded and further characterized. To identify MDCK cell clones with significant repression of PKA signaling, each clone was transiently transfected with a CRE-driven reporter construct (pCRE-SEAP; Clontech), followed by stimulation with forskolin (20 µM) for 24 h. From the 24 clones initially obtained in cells transfected with pCMV-KCCREB, 3-4 showed strongly reduced response to forskolin; these were pooled and used in further experiments. Pooled clones transfected with wild-type CREB were used as controls. For transient transfection assays, a total of 4 x 10^6 exponential MDCK cells were electroporated with 40 µg of the respective plasmids in 400 µl HEPES-buffered saline at 300 V and 950 µF in a 4-mm cuvette, using a Bio-Rad Gene Pulser Xcell apparatus.

Determination of COX activity.

COX activity in cell lysates was determined using a commercially available COX activity assay kit (Cayman Chemicals). The measurement is based on the detection of the peroxidase activity of COX, which is monitored colorimetrically by the formation of oxidized N, N, N′, N′-tetramethyl-p-phenylendiamine (TMPD) at 620 nm. Briefly, cells were washed with ice-cold TBS, harvested in 200 µl TBS containing 1 mM

EDTA and 1% (vol/vol) broad-specificity protease inhibitor cocktail (Sigma), and lysed mechanically by repeated vigorous passage through a 26-gauge needle. After centrifugation at 13000 g for 15 min at 4°C, COX activity was determined in equal volumes of the supernatant according to the manufacturer's protocol. To discriminate between COX-1 and COX-2 activity, each sample was compared with a corresponding one containing an isoenzyme-specific inhibitor for COX-1 (SC-560, 330 nM), and COX-2 (DuP-697, 300 nM), respectively. Subsequently, COX activity was normalized to the protein concentration in the cell lysate (expressed as U/µg protein).

PGE_2 measurement.

PGE_2 concentrations in 100-µl medium aliquots were determined using a commercially available assay (PGE_2 EIA Kit; Cayman Chemicals) according to the manufacturer's protocol. Plates were read at 405 nm in a plate reader, and PGE_2 concentrations were determined from the standard curve.

COX-2 promoter activity assay.

For construction of a canine COX-2 reporter vector, genomic DNA from MDCK cells was used as template. The 5'-flanking region reaching from bp −825 to +1 of the canine COX-2 gene was amplified by PCR, using primers 5′-GGTGGCCAAAGTGGTGAA-3′ (sense) and 5′-AGACGCTCGCTGCAAGTT-3′ (antisense), and subcloned into the pJET vector by use of the CloneJET PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's recommendations. The resulting plasmid pJET-COX-2-Promoter was cut with NcoI, blunted with T4 DNA polymerase, and subsequently cut with XhoI to mobilize the

fragment containing the COX-2 promoter region, which was subcloned into the pSEAP-basic vector (Clontech), using restriction sites XhoI and NruI. The sequence of the resulting plasmid pSEAP-COX-2-Promoter, containing the COX-2 promoter fragment (bp −825 to +1) upstream of a secreted alkaline phosphatase reporter gene, was verified by sequencing. A computer-based analysis of the COX-2 promoter region using the TESS algoritm (www.cbil.upenn.edu/cgi-bin/tess) revealed a putative CRE site at bp −78 to −74 (5′-CGTCA-3′) in the COX-2 promoter region. This site was deleted from the plasmid pSEAP-COX-2-Promoter of the first four nucleotides (CGTC) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers for the mutagenesis were 5′-GTGGAAAGAAACAGTCATG*ACGTGGGCTTAGTTATCAT-3′ (sense) and 5′-ATGATAACTAAGCCCACGT*CATGACTGTTTCTTTCCAC-3′ (antisense; the * marks the position where the four nucleotides were deleted). The mutagenesis of the CRE site was verified by sequencing the resulting plasmid pSEAP-COX-2 Promoter-CRM. MDCK cells were transfected with pSEAP-COX-2-Promoter and pSEAP-COX-2 Promoter-CRM using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Reporter gene assays were performed as described above.

Determination of caspase-3 activity.

Caspase-3 activity was assessed using an assay based on the cleavage of a synthetic p-nitroaniline-conjugated caspase-3 substrate (Asp-Glu-Val-Asp; CaspACE Assay System; Promega, Madison, WI). The cells were treated as indicated, subsequently washed with ice-cold PBS, and disrupted by the addition of 100 µl lysis reagent (Promega) per 35-mm dish, followed by three freeze-thaw cycles. After

centrifugation at 13,000 g for 20 min at 4°C, activity of caspase-3 was measured according to the manufacturer's recommendations using 20 µl supernatant. Caspase-3 activity was determined by increase in A_{405} and was normalized to the protein concentration of the cell lysates. Relative caspase-3 activity is expressed as picomoles p-nitroaniline liberated per microgram protein in 1 h.

Immunohistochemistry.

All experiments were conducted in accordance with German federal laws relating to animal experimentation. The kidneys of male Wistar rats (200–250 g) were cut into 2-mm slices along the cortico-medullary axis, fixed overnight in 4% paraformaldehyde in phosphate buffer, embedded in paraffin, and cut into 4-µm-thick sections. After pretreatment in a microwave for 3 x 5 min, endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Thereafter, the sections were rinsed with PBS, and nonspecific binding sites were blocked by incubation with 3% goat serum in PBS for 30 min at room temperature. Subsequently, sections were incubated with rabbit polyclonal COX-2 antiserum (Cayman Chemicals), diluted 1:600 in PBS overnight at 4°C, and subsequently with secondary biotin-conjugated goat anti-rabbit antiserum (1:200 in 3% goat serum in PBS). Immunocomplexes were visualized using an ABC-detection kit according to the manufacturer's protocol (Vectastain; Vector Laboratories) with diaminobenzidine tetrahydrochloride-0.1% H_2O_2 in PBS as chromogenous substrate. Finally, the sections were counterstained with hemalaun and mounted. In negative controls, the primary antibody was omitted.

Presentation of data and statistical analysis.

Data are presented as means ± SE. The significance of differences between means

was determined using Student's t-test, or ANOVA for multiple comparisons. $P < 0.05$ was regarded as significant.

Results

Effect of PGE_2 on tonicity-induced COX-2 expression.

The present study addressed the hypothesis that PGE_2 affects the expression of COX-2 via a feedback mechanism under hypertonic conditions and investigated the signaling pathways and functional consequence of this putative feedback loop in closer detail. In the first step, the time course of tonicity-induced COX-2 expression and the effect of PGE_2 were assessed. As demonstrated in Fig. 1, adding PGE_2 under hypertonic conditions caused an increase in COX-2 mRNA (Fig. 1A) and protein (Fig. 1B) levels. This effect became evident at PGE_2 concentrations of 5 μM and higher. Therefore, in the following experiments, the lowest effective PGE_2 concentration, i.e., 5 μM, was used. Adding PGE_2 to isotonic medium at even high concentrations (i.e., 20 μM) failed, however, to elicit any effect on COX-2 expression (Fig. 2A). In addition, the stimulatory effect of PGE_2 on COX-2 induction was also evident after raising the medium tonicity quasilinearly and gradually over several hours as occurs in the kidney in vivo following stimulation of the urinary concentrating mechanism (Fig. 2B).

Figure 3 shows the time course of COX-2 induction in response to osmotic stress in the absence and presence of PGE_2. COX-2 mRNA (Fig. 3A) and protein (Fig. 3B) levels were increased transiently, reaching a maximum after 6 and 24 h after elevating the medium tonicity, respectively. COX-2 induction was potentiated substantially in the presence of PGE_2 in the hypertonic medium.

Effect of PGE_2 on COX-2 activity.

To assess whether the observed changes in COX-2 expression on mRNA and protein levels are paralleled by alterations in enzyme activity, the latter was determined in cell lysates under the conditions shown in Fig. 4*A*. COX activity was increased significantly by hypertonicity and was further elevated significantly by PGE_2, an effect that was diminished by the protein kinase A (PKA) inhibitor H-89.

Addition of the specific COX-2 inhibitor DuP-678 reduced total COX activity in the samples by about 60% (Fig. 4B), whereas the selective COX-1 inhibitor SC-560 had only marginal effects, indicating that COX-2 is the major COX isoform accounting for COX activity during osmotic stress and that PGE_2 stimulates COX-2 activity during osmotic stress. To address whether a rise in cellular ionic strength may reduce COX-2 activity under hypertonic conditions, the assay buffer was adjusted with KCl to intracellular levels as observed during osmotic stress. We observed, however, no significant changes in COX activity in this approach, suggesting that an increase in cellular ionic strength does not significantly modulate COX activity (not shown).

To directly address whether the proposed PGE_2-cAMP pathway stimulates PGE_2 production, PGE_2 concentration in the medium was measured from MDCK cells incubated under isotonic conditions or under hypertonic conditions in the presence or absence of the cAMP mimetic dibutyryl-cAMP. As demonstrated in Fig. 5, osmotic stress increased PGE_2 release severalfold, which was further more than doubled by the addition of dibutyryl-cAMP. The reason why COX activity as measured in the activity assay does not increase to the same extent as PGE_2 production is unknown; however, this phenomenon may be due to the fact that COX activity is determined in cell lysates using an in vitro assay, which may not reflect accurately the environment inside of intact cells.

Signaling pathways involved in stimulation of COX-2 induction by PGE_2.

The next aim was to investigate potential signaling pathways for the observed positive feedback effect of PGE_2 on COX-2 expression during osmotic stress. Preliminary experiments indicated that a cAMP-dependent mechanism is involved. This is inferred from the observation that PKA inhibition drastically reduced cell survival during osmotic stress and the fact that PGE_2 substantially improves cell viability under hypertonic conditions, suggesting a mechanism that involves signaling through EP2 and / or EP4 receptors. This would be consistent with previous findings demonstrating stimulation of COX-2 expression in an EP2 receptor, PKA-dependent manner in a murine intestinal polyposis model (41), and prosurvival effects of PGE_2 following UVB irradiation, which were mediated by EP2 and / or EP4 receptors (6, 16). To address this issue during osmotic stress, MDCK cells were pretreated with the EP2 receptor antagonist AH-6809 or the EP4 antagonist AH-23848 before increasing the medium tonicity in the presence of PGE_2. As shown in Fig. 6, AH-6809 diminished the stimulatory effect on COX-2 induction at the mRNA (Fig. 6*A*) and protein (Fig. 6*B*) level, whereas no significant effect was evident in the presence of AH-23848. Accordingly, forskolin, an activator of adenylate cyclase, and the cAMP mimetic dibutyryl-cAMP mimicked the stimulatory effect of PGE_2 on COX-2 expression, thus supporting the notion that this effect is mediated by an EP2-dependent increase in intracellular cAMP. In agreement, the response to PGE_2 was abolished by H-89, an inhibitor of PKA. To confirm the expression of players in this signaling pathway in MDCK cells, immunoblot analyses for EP2 and CREB were performed. As shown in Fig. 6*C*, both the putative upstream (EP2) and downstream (CREB) signaling molecules are expressed in these cells under isotonic and hypertonic conditions. Accordingly, the presence of functional cAMP-PKA signaling in

response to PGE_2 has been demonstrated recently (33).

Role of the CREB-CRE pathway in stimulation of COX-2 induction by PGE_2.

The above observations are compatible with the view that the positive feedback effect of PGE_2 on COX-2 is conveyed by PKA-mediated activation of CREB with subsequent stimulation of CRE, thereby enhancing COX-2 transcription. Particularly, signaling through both NF-κB and CREB is involved in COX-2 induction in response to various stimuli (8, 23, 44, 47). To identify the pathway involved in the present study, activation of these signaling pathways was assessed in MDCK cells stably transfected with reporter gene constructs driven by NF-κB or CRE sites, respectively. CRE-driven reporter activity under the individual conditions is shown in Fig. 7A. In the absence of PGE_2, no difference in CRE-driven reporter activity was evident by raising the medium osmolality from 300 to 500 mosmol/kg H_2O. PGE_2, added under isotonic conditions, enhanced reporter activity about fivefold, whereas addition of PGE_2 to hypertonic medium increased reporter activity 15- to 17-fold compared with isotonic controls. In general, reflecting the effects observed on COX-2 expression, CRE-driven reporter activity was not affected by AH-23848 but diminished by the EP2 antagonist AH-6809 and the PKA inhibitor H-89. Forskolin, as well as dibutyryl-cAMP, enhanced reporter activity comparably to PGE_2 (Fig. 7A). In contrast to the CRE-driven construct, hyperosmolality in the absence or presence of PGE_2 caused no significant changes in reporter activity in MDCK cells transfected with a reporter construct under the control of a NF-κB site (Fig. 7B). Although COX-2 is not a known TonEBP target gene, the impact of PGE_2 on TonE-stimulated reporter activity was additionally investigated. In MDCK cells stably transfected with a TonE-driven reporter vector, hypertonicity elicited a robust increase in reporter gene expression,

as expected; however, this response was not further stimulated by PGE_2 (Fig. 7C).

To directly address the stimulatory effect of PGE_2 on COX-2 expression at the promoter level, a portion of the canine COX-2 promoter was cloned from MDCK cells. In the resulting construct, the SEAP gene is driven by a −825 to +1 canine COX-2 promoter fragment. Under hypertonic conditions, reporter activity was only moderately increased compared with isotonic conditions, whereas addition of PGE_2 further significantly enhanced reporter activity (Fig. 8A). The reason for the minor degree of induction compared with the induction at the mRNA and protein level may be caused by regulatory elements upstream of −825 that are required for full transcriptional activation during osmotic stress. Accordingly, reporter activity under hypertonic conditions in the present experiments was in the same range as in a previous study by Yang et al. (49).

A database search revealed the presence of a putative CRE at bp −78 to −74 in the canine COX-2 promoter. To investigate the contribution of this CRE to the stimulatory effect of PGE_2 on COX-2 expression, the respective sites were deleted by site-directed mutagenesis. In MDCK cells transfected with the CRE-deleted construct, reporter activity was elevated to the same extent as with the wild-type promoter under hypertonic conditions, whereas no further increase was evident in the presence of PGE_2 (Fig. 8B). These observations suggest that CRE signaling mediates the stimulatory effect of PGE_2 on COX-2 induction during osmotic stress.

In summary, the above findings favor a cAMP-PKA-CREB-dependent pathway that mediates enhanced COX-2 expression in the presence of PGE_2. To test this hypothesis directly, MDCK cells were transfected with wild-type or dominant-negative CREB (KCREB) (45). As demonstrated in Fig. 9A, in cells transfected with wild-type CREB, tonicity-induced COX-2 protein expression was further significantly increased

by PGE_2. This effect was however abolished in MDCK cells transfected with dominant-negative CREB, further strengthening the concept that the stimulatory effect of PGE_2 likely relies on an EP2-cAMP-PKA-CREB-dependent pathway. The latter experiments were performed with stably transfected MDCK cells. To exclude any bias by selecting and expanding single cell clones, the experiment was repeated in transient transfection assays, i.e., MDCK cells were electroporated with the respective expression constructs. Immunoblot analysis with an antibody that detects both wild-type CREB and KCREB showed substantially higher immunoreactivity in electroprated cells compared with nontransfected cells, suggesting efficient gene delivery (Fig. 9C). Regarding COX-2 expression under the various experimental conditions, essentially the same results were obtained as in stably transfected cells (Fig. 9B), supporting the notion that CREB-CRE signaling indeed mediates the positive feedback effect of PGE_2 on COX-2.

Effect of PGE_2 on biochemical indices of apoptosis during osmotic stress.

MDCK cell were subjected to hypertonic medium (650 mosmol/kg H_2O by NaCl addition) for 12 h. After this period, the cells were processed for measurement of caspase-3 activity. As demonstrated in Fig. 10, caspase-3 activity was 8- to 10-fold higher under hypertonic conditions than in cells kept at 300 mosmol/kgH_2O. Tonicity-induced increase in caspase-3 activity was however reduced significantly in the presence of PGE_2, forskolin, or dibutyryl-cAMP. Conversely, EP2 antagonism with AH-6809 as well as H-89 diminished the protective effect of PGE_2, whereas the EP4 antagonist AH-23848 had no significant effect (Fig. 10).

Phosphorylation of pro-apoptotic Bad by PGE_2.

Bad is a pro-apoptotic member of the Bcl-2 family that promotes cell death by binding to and inactivating anti-apoptotic Bcl-xL and Bcl-2. This pro-apoptotic activity of Bad is inhibited by PKA-mediated phosphorylation at Ser155 (9, 27, 53). Because phosphorylation at Ser155 disrupts the binding of Bad to pro-survival Bcl-2 proteins, thereby blocking apoptosis, this event might provide a potential explanation for the protective effect of PGE_2 during osmotic stress. To address this hypothesis, phosphorylation of Bad at Ser155 was assessed by immunoblot analysis. As evident from Fig. 11, Ser155-phosphorylated Bad protein was readily detectable in cells exposed to elevated ambient tonicity, but phosphorylation was considerably higher when PGE_2 was added to the hypertonic medium. This effect was mimicked by forskolin and blunted by the PKA inhibitor H-89. Total Bad protein abundance appears to be unchanged during the several treatments. In summary, PGE_2 promotes Bad phosphorylation at Ser155 in a cAMP-PKA-dependent pathway, which correlates with reduced caspase-3 activity during osmotic stress.

Expression of COX-2 in the renal medulla.

Although MDCK cells are frequently used as a model system for medullary collecting duct cells, the expression of COX-2 in the latter cells has been inconsistently demonstrated in the literature. To confirm the physiological relevance of the findings presented in the previous sections, intramedullary COX-2 expression was determined by immunohistochemistry on the kidneys of adult Wistar rats with free access to water. In the renal papilla, COX-2 immunoreactivity was most prominent in medullary interstitial cells, whereas inner medullary collecting duct (IMCD) cells exhibited no or only very faint staining (Fig. 12, A and B). These findings are

consistent with previous studies addressing the cellular localization of COX-2 in the renal inner medulla (13, 50), although COX-2 expression was also detected in IMCD cells in water-deprived rats (49). In the outer medulla, COX-2 immunoreactivity was localized specifically in outer medullary collecting duct cells, whereas other tubular epithelial cells and interstitial cells showed no or only minimal COX-2 immunoreactivity (Fig. 12, C and D). Immunostaining for COX-2 was also evident in the initial part of the IMCD (Fig. 12C). Thus the MDCK cell model that we used in the present study appears at least adequate in the context of OMCD cells, in addition, a functional relevance of the proposed positive feedback effect of PGE_2 on COX-2 expression is conceivable in IMCD cells.

Discussion

COX-derived prostanoids exert multiple essential functions within the kidney. In the renal medulla, locally produced PGE_2 reduces sodium and water reabsorption in the medullary thick ascending limb and the collecting duct, respectively, effects that are mediated in an autocrine/paracrine manner (18, 21). Moreover, PGE_2 dilates descending vasa recta, thereby buffering the constrictor effects of ANG II, AVP, and catecholamines during physiological stress, an action that is vitally important to prevent hypoxic damage to renal medullary cells (39). In contrast to most other tissues, COX-2 is constitutively expressed in the renal medulla and is induced further by dehydration when the urinary concentrating mechanism is stimulated (49). The relevance of renomedullary COX-2-derived PGE_2 is underscored by the observation that selective COX-2 inhibitors are associated with renal side effects similar to those observed with traditional nonselective nonsteroidal anti-inflammatory drugs (14, 40).

We have demonstrated recently that PGE_2 favors the adaptation of renal medullary cells to elevated ambient tonicities as present in the renal papilla during antidiuresis (25, 37). In addition to the enhanced expression of several osmosensitive genes (37), COX-2 was induced even more vigorously in response to hypertonicity in the presence of PGE_2 following a nearly gradual increase in ambient tonicity (37). Consistent with this finding, corresponding observations were evident in the present experiments, where a step increase in extracellular osmolality was employed (Figs. 1 and 2). In aggregate, these findings confirm the existence of a positive feedback loop of PGE_2 on COX-2 expression during osmotic stress. The present study thus aimed to characterize this phenomenon in more detail and to delineate the underlying signaling pathways and the functional relevance of this observation.

PGE_2 substantially increased tonicity-induced COX-2 mRNA and protein expression and activity, an effect that appears to depend on signaling through EP2 receptors with subsequent formation of cAMP and activation of PKA (Figs. 1–3). Both types of $G_{\alpha s}$-coupled EP receptors, i.e., EP2 and EP4, have been detected in MDCK cells (26, 38). Although the precise cellular localization of EP2 receptors in the kidney is not well defined, EP2 mRNA has been detected in the inner and outer medulla of the rat kidney but not in the cortex (19, 20). Furthermore, mice with targeted disruption of the EP2 receptor develop salt-sensitive hypertension, suggesting that EP2 plays an important role in the renal medulla (22). In contrast to the intrarenal distribution of EP2, EP4 is expressed abundantly in glomeruli but is not detected in the renal medulla (5). Consistently, in the present study, both the EP2 antagonist AH-6809 and the PKA inhibitor H-89 blunted the stimulatory effect of PGE_2 on COX-2 expression, whereas the EP4 inhibitor AH-23848 had no significant effect (Fig. 6). Conversely, the cAMP mimetics forskolin and dibutyryl-cAMP potently enhanced COX-2 expression under hypertonic conditions (Fig. 6). However, we did not address the effect of EP2 or EP4 inhibition in the absence of exogenously added PGE_2 in the present study nor did we examine the effect selective COX-2 inhibitors. Thus we cannot delineate the role of endogenously produced PGE_2 in MDCK cells nor can be completely excluded that alternative pathways or interactions with members of other signaling cascades are involved in the observed effects. cAMP exerts a multitude of biological actions and additional effectors of cAMP other than PKA have been identified (15). Thus our primarily inhibitor-based approach does not completely rule out the possibility that signaling events distinct from the classical G_s-coupled receptor-cAMP-PKA-CREB pathway contribute to the stimulatory effect of PGE_2 on COX-2 expression during osmotic stress. Nevertheless, the most likely explanation

for the observed effects is activation of the classical cAMP-PKA signaling cascade, which is in agreement with previous findings in a murine intestinal polyposis model (41). In the latter investigation, COX-2 expression was boosted by PGE_2, acting through EP2 receptors and elevation in intracellular cAMP levels in a positive feedback loop (41).

Although the present data support a role of cAMP-PKA signaling in COX-2 expression and cell survival under hypertonic conditions, other studies failed to detect activation of PKA in IMCD cells (28) and stimulation of cAMP production in HepG2 cells (10) in response to hypertonicity. Nevertheless these findings are consistent with the present observations that osmotic stress only negligibly increases CRE-driven reporter activity in the absence of exogenous PGE_2, whereas the latter is robustly enhanced in the presence of PGE_2 (Fig. 7A). These observations may either relate to different cell types used, or even of greater importance, that PGE_2 may be more readily available for renal medullary cells in an auto-/paracrine manner in the local cellular environment in the renal medulla in situ and that cell culture systems represent a simplification of the complex in vivo situation. Although MDCK are frequently used to address cellular and molecular aspects in osmoadaptation of renal medullary cells, their precise origin is unknown, which may represent a limitation of the present study. In addition, we did not evaluate the effect of endogenously produced PGE_2 or the effect of EP2 and EP4 inhibitors in the absence of exogenous PGE_2, which may represent a further limitation.

Several consensus elements including those for NF-κB and CRE have been identified as regulatory sequences in the promoter region of COX-2 from various cell types from different species that are involved in COX-2 induction during cell stress (47, 48). In MDCK cells transfected with a CRE-driven reporter construct, reporter

gene expression closely correlated with COX-2 abundance under the various experimental conditions (Fig. 6A), which was not the case in cells transfected with a reporter construct under control of a NF-κB site (Fig. 6B). A functional contribution of CREB-CRE signaling is further supported by the observation that deletion of a potential CRE in the canine COX-2 promoter abolishes the stimulatory effect of PGE_2 (Fig. 8), which was also the case after transfection with dominant-negative CREB (Fig. 9). Interestingly, under isotonic conditions, PGE_2 was not sufficient to enhance COX-2, despite robust induction of CRE-driven reporter activity (Fig. 7A). These observations indicate that additional signaling pathways that are activated during osmotic stress (i.e., p38, ERK1/2) are required for stimulation of COX-2 expression under hypertonic conditions, as previously suggested by findings of our group and others (25, 52).

Furthermore, PGE_2 significantly attenuated biochemical indices of apoptosis as shown for caspase-3 activity in MDCK cells exposed to tonicities that are found routinely in medulla of the concentrating kidney (3). Again, this effect could be blunted by EP2, but not EP4 antagonism, and by PKA inhibition, whereas the effect of PGE_2 could be reproduced by forskolin and dibutyryl-cAMP (Fig. 10), supporting the existence of an EP2-cAMP-PKA-dependent pro-survival mechanism that is activated by PGE_2. Interestingly, a corresponding mechanism has been described recently in gamma radiation-mediated apoptosis in mouse intestine and UVB-induced apoptosis in mouse skin (6, 16). Notably, both irradiation and hyperosmolality entail structural DNA damage, including double-strand breaks (11). In UVB-induced apoptosis in mouse skin, COX-2-derived PGE_2 inhibited apoptosis via an EP2-cAMP-PKA-dependent pathway, a response mediated by phosphorylation of the pro-apoptotic protein Bad (6). Bad belongs to a family of proteins regulating survival

factor / apoptosis checkpoints in the cellular death machinery, which either promote survival (e.g., Bcl-2, Bcl-xL), or apoptosis (e.g., Bad, Bak) (9). Activated PKA phosphorylates Bad at Ser155, which inactivates the pro-apoptotic function of Bad by impeding the cytotoxic interaction of Bad with anti-apoptotic Bcl-2 and Bcl-x_L proteins at the mitochondrial membrane (9, 53). This mechanism is of particular importance for survival factors acting through their cognate cell surface receptors and appears to be crucial in epithelial cells (24, 43). In MDCK cells, overexpression of Bad has been shown to increase caspase-3 activity, which was diminished by phosphorylation of Bad (17). These findings are in good agreement with the present results demonstrating that Bad is phosphorylated at Ser155 in the presence of PGE_2 and forskolin, known activators of PKA, whereas PGE_2-induced Bad phosphorylation is prevented by PKA inhibition with H-89 (Fig. 11). Notably, Bad phosphorylation was associated with reduced caspase-3 activity during osmotic stress, whereas pharmacological inhibition of the EP2-cAMP-PKA axis significantly increased caspase-3 activity (Fig. 8). Although the present data are consistent with an anti-apoptotic mechanism related to phosphorylation of Bad, they do not establish a causal role of the proposed mechanism in the present model.

In conclusion, the present study demonstrates that the major medullary prostanoid, PGE_2, increases tonicity-induced COX-2 expression and activity in a positive feedback loop that likely involves signaling through a PGE_2-EP2-cAMP-PKA-CREB-CRE-dependent pathway. In addition, activation of this mechanism prevents biochemical indices of apoptosis during osmotic stress, suggesting that this pathway is relevant during osmoadaptation of renal medullary cells. Finally, PGE_2 causes PKA-dependent phosphorylation of pro-apoptotic Bad, coinciding with reduced

apoptotic indices, thereby providing a possible mechanistic link for the prosurvival effect of PGE_2 under hypertonic conditions.

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Legends for figures

Figure 1. Effect of prostaglandin E_2 (PGE_2) on tonicity-induced COX-2 expression.

MDCK cells were incubated in isotonic (300 mosmol/kg H_2O, *iso*) or hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*) in the presence of 1, 5, 10, or and 20 µM PGE_2, or only vehicle ethanol (*vehicle*). (A) COX-2 mRNA abundance was assessed after 6 h incubation by Northern blot analysis. COX-2 mRNA expression was normalized to that of GAPDH. Representative blots are shown. Means±SEM for *n*=3; * *P*<0.05 vs. *NaCl* and *NaCl+vehicle*. (B) COX-2 protein expression was determined after 24 h by Western blot analysis. To demonstrate equal protein loading, the membranes were also stained for actin. Representative blots are shown. Means±SEM for *n*=4-10; * *P*<0.05 vs. *NaCl* and *NaCl+vehicle*.

Figure 2. Effect of PGE_2 on COX-2 expression under isotonic conditions and following a gradual tonicity increase.

(A) MDCK cells were exposed to isotonic medium (300 mosmol/kg H_2O) in the presence of 20 µM PGE_2 (*iso+PGE_2*), or vehicle ethanol (*iso+vehicle*). The membrane was reprobed for GAPDH to demonstrate comparable mRNA loading. Representative blots including 4 independent experiments are shown. (B) MDCK cells were exposed to isotonic medium (300 mosmol/kg H_2O, *iso*) or to medium in which the osmolality was raised quasi-linearly and gradually to 700 mosm/kgH_2O over a period of 4 h by repeated addition of NaCl in presence of PGE_2 (*NaCl+PGE_2*) or vehicle ethanol (*NaCl+vehicle*). After an additional 6 h, COX-2 mRNA expression was determined by Northern blot analysis. The signal for COX-2 was normalized to

GAPDH to correct for differences in RNA loading. Means±SEM for *n*=4; * *P*<0.05 vs. *iso*; # *P*<0.05 vs. *NaCl+vehicle*; Representative blots are shown.

Figure 3. Time course of COX-2 expression following osmotic stress.

MDCK cells were exposed to hypertonic medium (500 mosm/kg H_2O by NaCl addition) for the indicated periods in the presence of 5 µM PGE_2 (*NaCl+PGE₂*) or only vehicle ethanol (*NaCl+vehicle*). (A) COX-2 mRNA abundance was determined by Northern blot analysis. COX-2 mRNA expression was normalized to that of GAPDH to correct for differences in RNA loading. Representative blots from two independent experiments are shown. (B) COX-2 protein expression was determined by Western blot analysis. The membranes were also stained for actin to demonstrate equal protein loading. Representative blots are shown. Means±SEM for *n*=4; * *P*<0.05 vs. *NaCl+vehicle*.

Figure 4. Effect of PGE_2 on COX activity under hypertonic conditions.

(A) MDCK cells were kept in isotonic medium (300 mosm/kgH_2O, *iso*) or in hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*) in the presence of 5 µM PGE_2 (*PGE_2*), or vehicle ethanol (*vehicle*). In some experiments, the cells were pretreated with H-89 (20 µM) prior to addition of 5 µM PGE_2 under hypertonic conditions (*PGE_2+H-89*). After 24 h, COX-2 activity was determined as described in Methods. Means±SEM for *n*=3-4; § *P*<0.05 vs. *iso*; * *P*<0.05 vs. *NaCl* and *NaCl+vehicle; # P*<0.05 vs. *NaCl+PGE_2*. (B) COX isoform activity from cells kept in hypertonic medium (500 mosm/kgH_2O) in the presence of 5 µM PGE_2 was assessed by addition of an iso-enzyme specific inhibitor for COX-1 (*SC-560)* and COX-2 (*DuP-*

697), respectively, to differentiate COX-1 from COX-2 activity. Means±SEM for *n*=4; * *P*<0.05 vs. *SC-560*.

Figure 5. Effect of cAMP signaling on PGE_2 production under hypertonic conditions.
MDCK cells were kept in isotonic medium (300 mosmol/kg H_2O, *iso*) or in hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*) in the presence of 1 mM dibutyryl-cAMP 5 µM PGE_2 (*cAMP*), or vehicle PBS (*vehicle*). After 24 h, PGE_2 concentration in the medium was determined as described in Methods. Means±SEM for *n*=4; * *P*<0.05 vs. *iso; # P*<0.05 vs. *NaCl+vehicle*.

Figure 6. Signaling pathways involved in PGE_2-mediated COX-2 stimulation during osmotic stress.
MDCK cells were incubated in isotonic medium (300 mosmol/kg H_2O, *iso*) or in hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*). Where indicated, the cells were exposed to 5 µM PGE_2 (*PGE_2*) or vehicle ethanol (*vehicle*) under hypertonic conditions. In experiments with pharmacological inhibitors, the cells were pretreated with 30 µM AH23848 (*PGE_2+AH23848*), 10 µM AH6809 (*PGE_2+AH6809*), or 20 µM H-89 (*PGE_2+H-89*), respectively, prior to adding PGE_2. In some experiments, the cells were treated with 10 µM forskolin (*forskolin*), or 1mM dibutyryl-cAMP (*dibutyryl-cAMP*) under hypertonic conditions as indicated. (A) COX-2 mRNA was determined after 6h incubation by Northern blot analysis. COX-2 mRNA abundance was normalized to that of GAPDH. Representative blots are shown. Means±SEM for *n*=2. (B) After 24 h incubation, COX-2 protein expression was assessed by Western blot analysis. To demonstrate equal protein loading, the membranes were also stained for actin. Representative blots are shown.

Means±SEM for *n*=5-10; * *P*<0.05 vs. *NaCl; # P*<0.05 vs. *PGE_2*. (C) Expression of EP2 and CREB in MDCK cells under isotonic (*iso*) or hypertonic conditions (*NaCl*). Representative blots are shown.

Figure 7. Effect of PGE_2 on CRE, (NF)-κB, and TonE reporter activity.

MDCK cells transfected stably with reporter constructs driven by cAMP-responsive element (CRE), nuclear factor-kappa B (NF-κB), or tonicity-responsive element (TonE) sites were treated for 24 h as indicated. Subsequently, relative reporter activity was determined as described in Methods. (A) MDCK cells, stably transfected with a CRE-driven reporter construct were exposed to isotonic medium (300 mosmol/kg H_2O, *iso*) or hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*) in the presence of 5 μM PGE_2 (*PGE_2*) or vehicle ethanol (*vehicle*). In experiments with pharmacological inhibitors, AH23848 (30 μM), AH6809 (10 μM), or H-89 (20 μM) were added 30 min prior to addition of PGE_2. In some experiments, the cells were treated with 10 μM forskolin (*forskolin*), or 1mM dibutyryl-cAMP (*dibutyrylcAMP*) under hypertonic conditions as indicated. Means±SEM for *n*=2-5; * *P*<0.05 vs. *NaCl; # P*<0.05 vs. *NaCl+PGE_2*. (B) MDCK cells, stably transfected with a NF-κB-driven reporter construct, were incubated in isotonic (*iso*) or hypertonic (500 mosmol/kg H_2O by NaCl addition) conditions in presence of 5 μM PGE_2 (*NaCl+PGE_2*) or vehicle ethanol (*NaCl+vehicle*). As positive control, the cells were exposed to 100 ng / ml tumor necrosis factor-α (*TNF-α*). Means±SEM for *n*=3-6; * *P*<0.05 vs. all other conditions. (C) MDCK cells, stably transfected with a TonE-driven reporter construct were incubated in isotonic (*iso*) or hypertonic (500 mosmol/kg H_2O by NaCl addition) medium in the presence of 5 μM PGE_2 (*NaCl+PGE_2*) or vehicle ethanol (*NaCl+vehicle*). Means±SEM for *n*=4; * *P*<0.05 vs. *iso.*

Figure 8. Stimulation of COX-2 promoter reporter activity by CRE signaling.

(A) MDCK cells were transfected with reporter vector under control of a -825 to +1 fragment of the canine COX-2 promoter. Subsequently, the cells were incubated for 24 h in isotonic (*iso*) or hypertonic medium (500 mosmol/kg H_2O by NaCl addition) in the presence of 5 µM PGE_2 (*NaCl+PGE$_2$*) or only vehicle ethanol (*NaCl+vehicle*) prior to determination of reporter activity. Means±SEM for *n*=6-10; * *P*<0.05 vs. *NaCl+vehicle*. (B) MDCK cells were transfected with the COX-2 promoter-driven reporter construct as described in (A), in which a putative CRE at bp -78 to -74 was deleted. Subsequently, the cells were treated as in (A) and processed for analysis of reporter activity. Means±SEM for *n*=6-10.

Figure 9. Effect of dominant-negative CREB on stimulation of tonicity-induced COX-2 expression by PGE$_2$.

(A) MDCK cells stably transfected with wild-type cAMP responsive element binding protein (*CREB*) or dominant-negative CREB (*KCREB*) were incubated in isotonic (300 mosm/kgH_2O, *iso*) or hypertonic medium (500 mosmol/kg H_2O by NaCl addition) in the presence of 5 µM PGE_2 (*NaCl+PGE$_2$*) or vehicle ethanol (*NaCl+vehicle*). After 24 h, COX-2 expression was determined by Western blot analysis. To demonstrate equal protein loading, the membranes were also probed for actin. Representative immunoblots are shown. Means±SEM for *n*=3-4; * *P*<0.05 vs. *NaCl*. (B) MDCK cells were electroporated with expression vectors for CREB and KCREB as described in methods. 24 h after electroporation, the cells were treated and processed as described in (*A*). Means±SEM for *n*=2. **P*<0.05 vs. *NaCl+vehicle*. (C) Representative Western blot analyses for expression of CREB in wild-type MDCK cells, and in MDCK cells electroporated with CREB or KCREB expression vectors.

Figure 10. Effect of PGE_2 on tonicity-induced activation of caspase-3.

MDCK cells were kept in isotonic medium (300 mosmol/kg H_2O, *iso*) or exposed to hypertonic medium (650 mosmol/kg H_2O by NaCl addition) in the presence of 5 µM PGE_2 (*PGE_2*) or vehicle ethanol (*vehicle*). In experiments with PGE_2, the cells were also pretreated with 30 µM AH23848 (*PGE_2+AH23848*), 10 µM AH6809 (*PGE_2+AH6809*), or 20 µM H-89 (*PGE_2+H-89*), respectively, prior to adding PGE_2. In some experiments, the cells were exposed to 10 µM forskolin (*forskolin*), or 1 mM dibutyryl-cAMP (*dibutyryl-cAMP*) under hypertonic conditions as indicated. After 12 h, caspase-3 activity was determined as described in Methods and expressed as pmol pNA liberated per hour per µg protein. Means±SEM for *n*=3-4; * *P*<0.05 vs. *PGE2;* # *P*<0.05 vs. *NaCl* and *vehicle*.

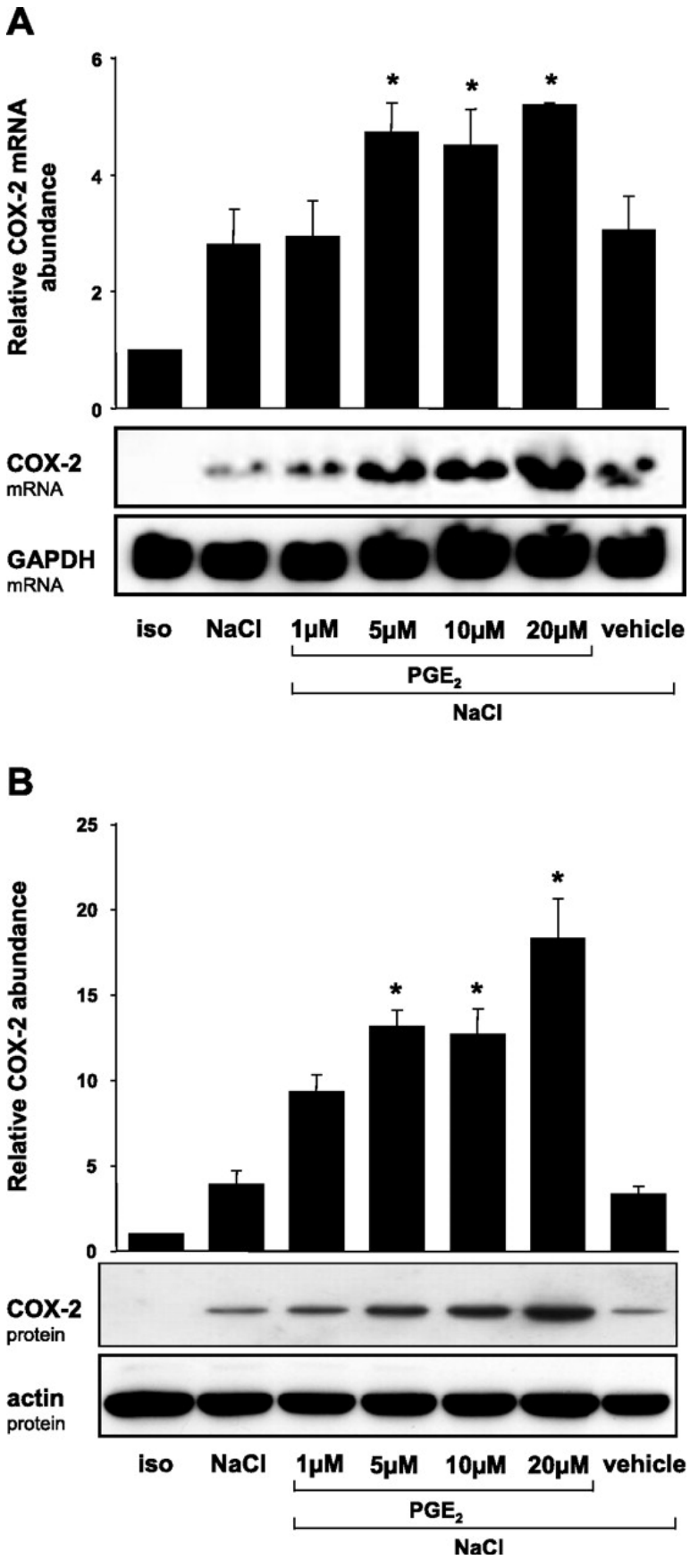
Figure 11. Effect of PGE_2 on phosphorylation of pro-apoptotic Bad protein.

MDCK cells were incubated in isotonic (300 mosmol/kg H_2O, *iso*) or hypertonic medium (500 mosmol/kg H_2O by NaCl addition, *NaCl*) in the presence of 5 µM PGE_2 (*PGE_2*), 10 µM forskolin (*forskolin*), or only vehicle ethanol (*NaCl*). Additionally, the cells were pretreated with 20 µM H-89 prior to addition of 5 µM PGE_2 under hypertonic conditions (*PGE_2+H-89*). After 24 h, total Bad expression, Bad phosphorylated at Ser155, and actin abundance was monitored by Western blot analysis using specific antibodies. Representative blots of 3 independent experiments are demonstrated.

Figure 12. Immunohistochemical localization of COX-2 in the inner (A, B) *and outer* (C, D) *medulla of the rat kidney.*

COX-2 immunoreactivity is most prominent in interstitial cells in the inner medulla (B) and in collecting duct cells in the outer medulla (D). Bars in A and C represent 200 μm and in B and D 100 μm.

Figures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PGE2
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Relative COX-2 abundance
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PGE2
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Figure 1

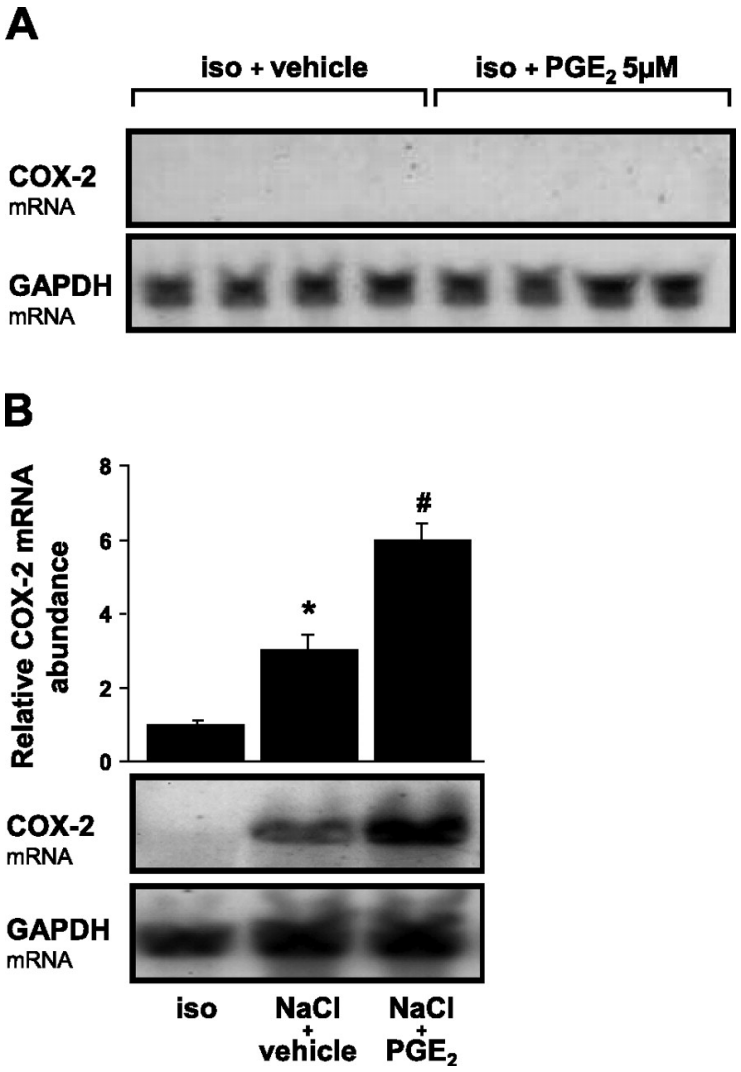
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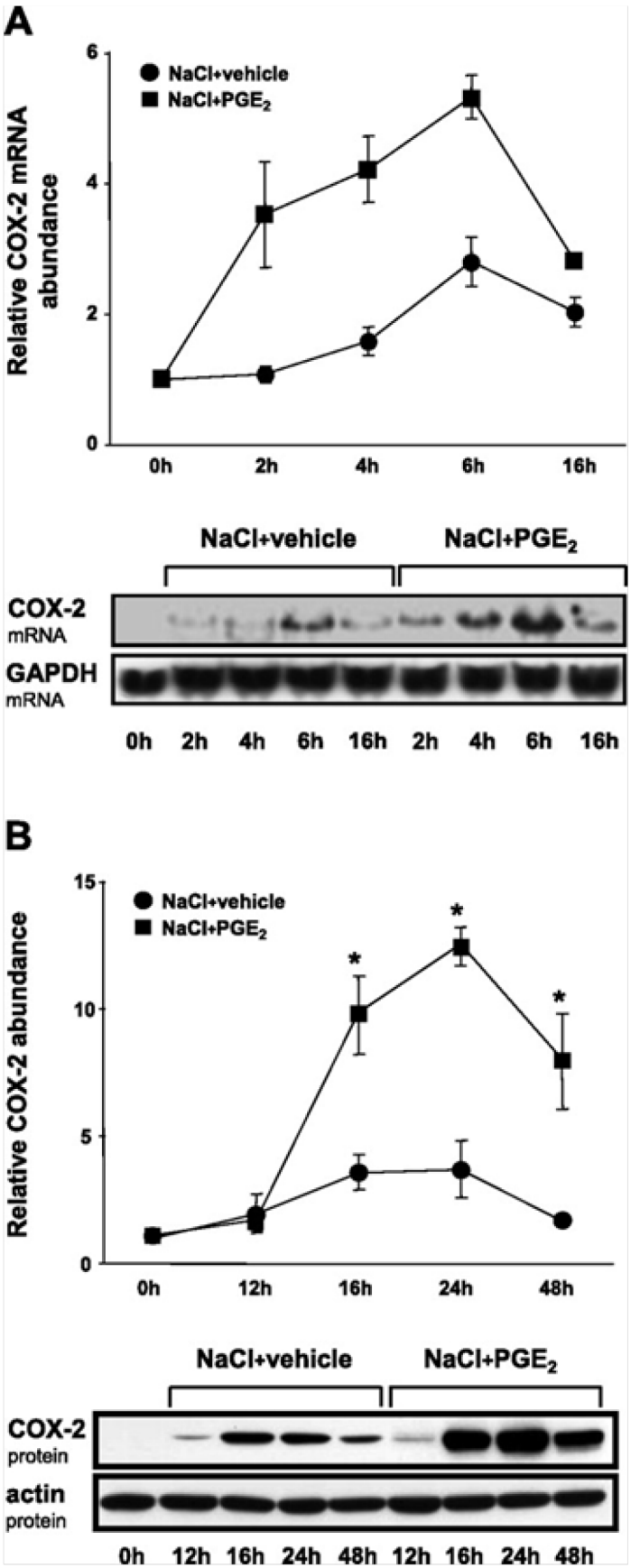
Figure 2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mRNA
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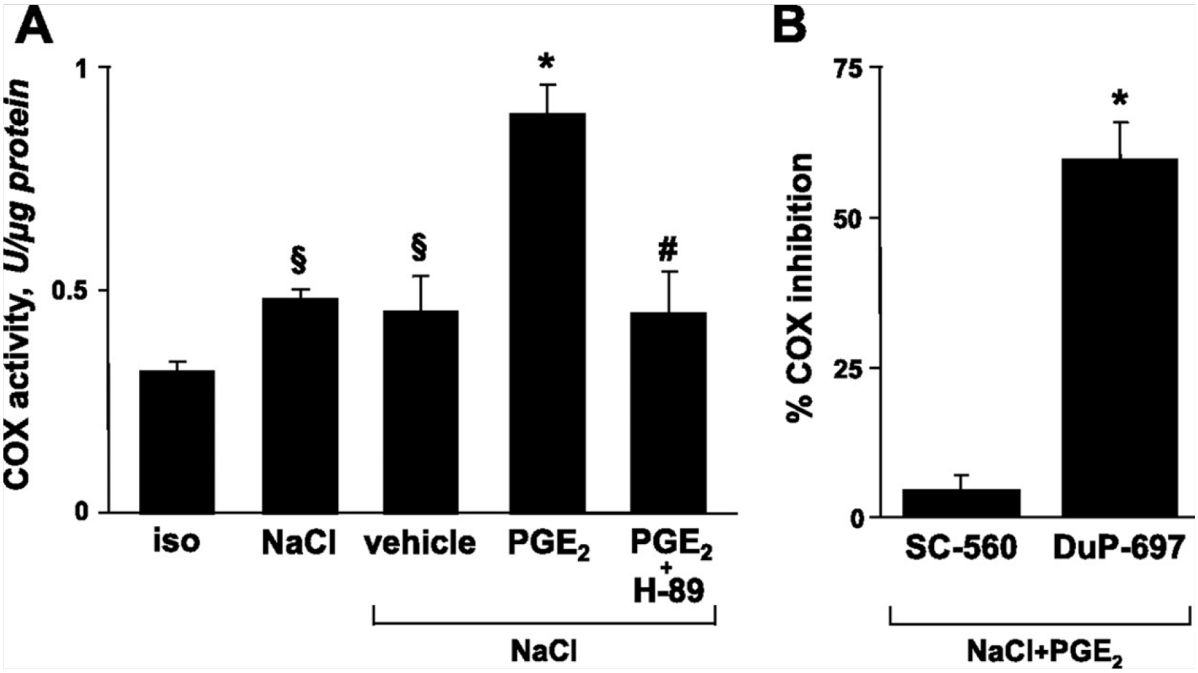
Figure 3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SC-560
DuP-697
NaCl+PGE2

Figure 4

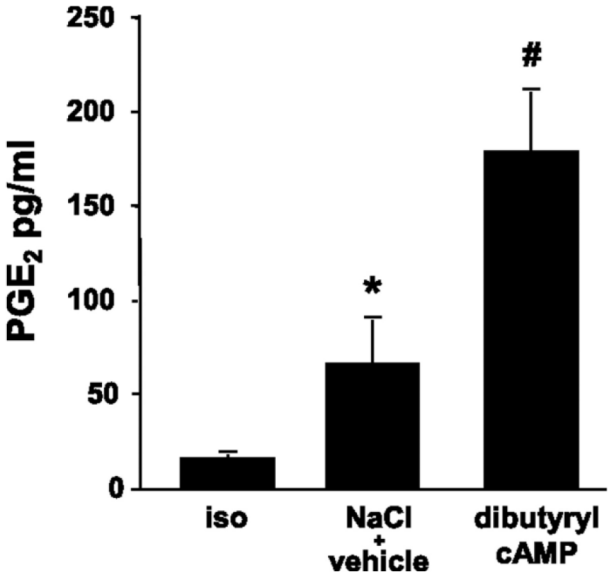
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Figure 5

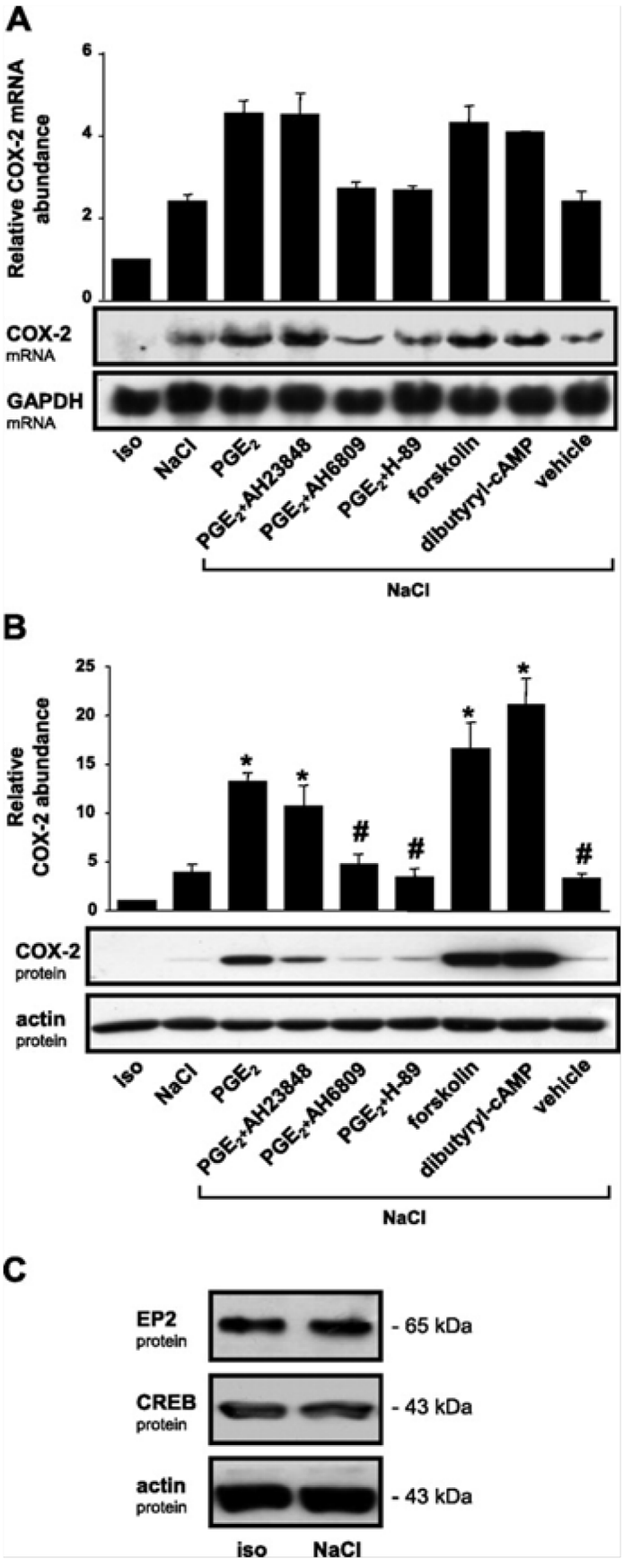
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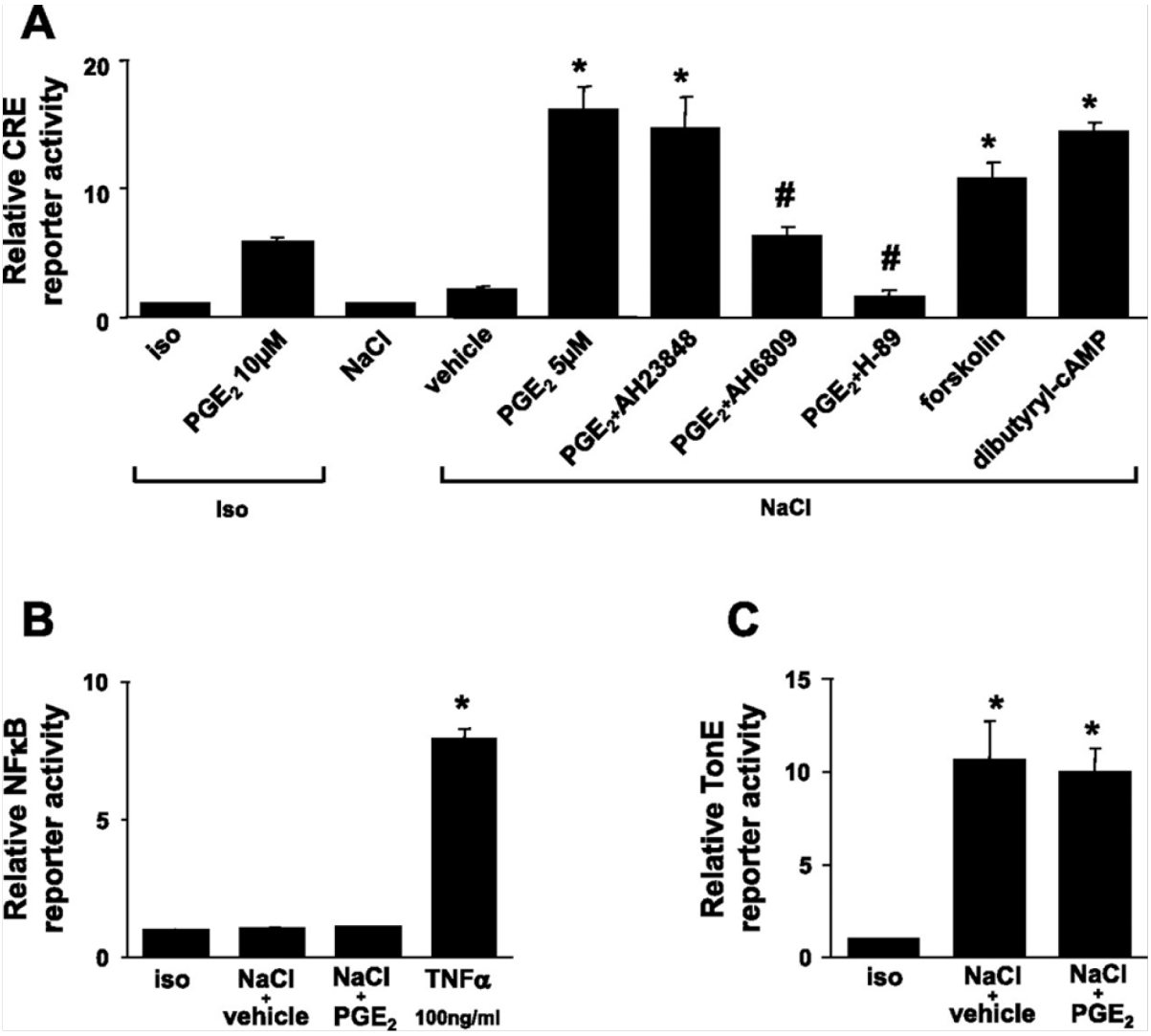
Figure 6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PGE2+AH6809
PGE2+H-89
forskolin
dibutyryl-cAMP
Iso
NaCl
B
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5
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*
iso
NaCl + vehicle
NaCl + PGE2
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C
Relative TonE reporter activity
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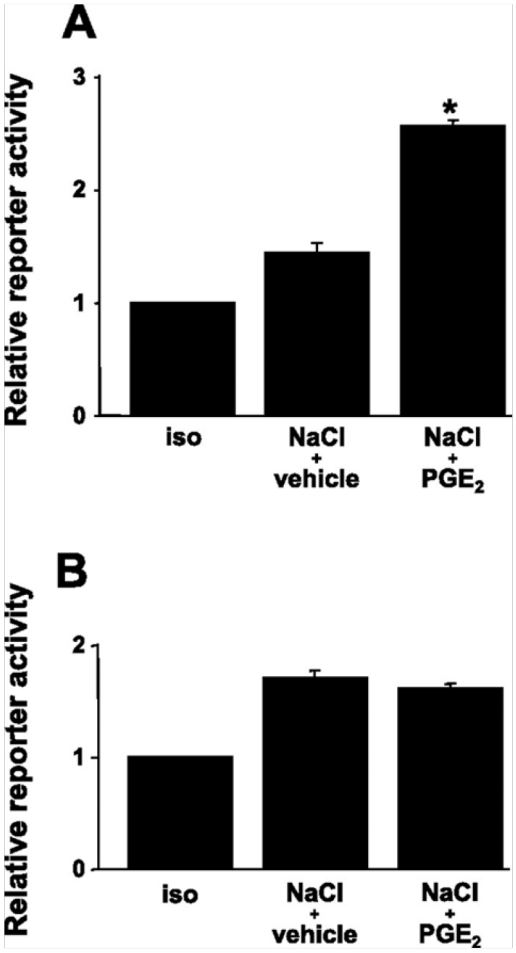
iso
NaCl + vehicle
NaCl + PGE2

Figure 7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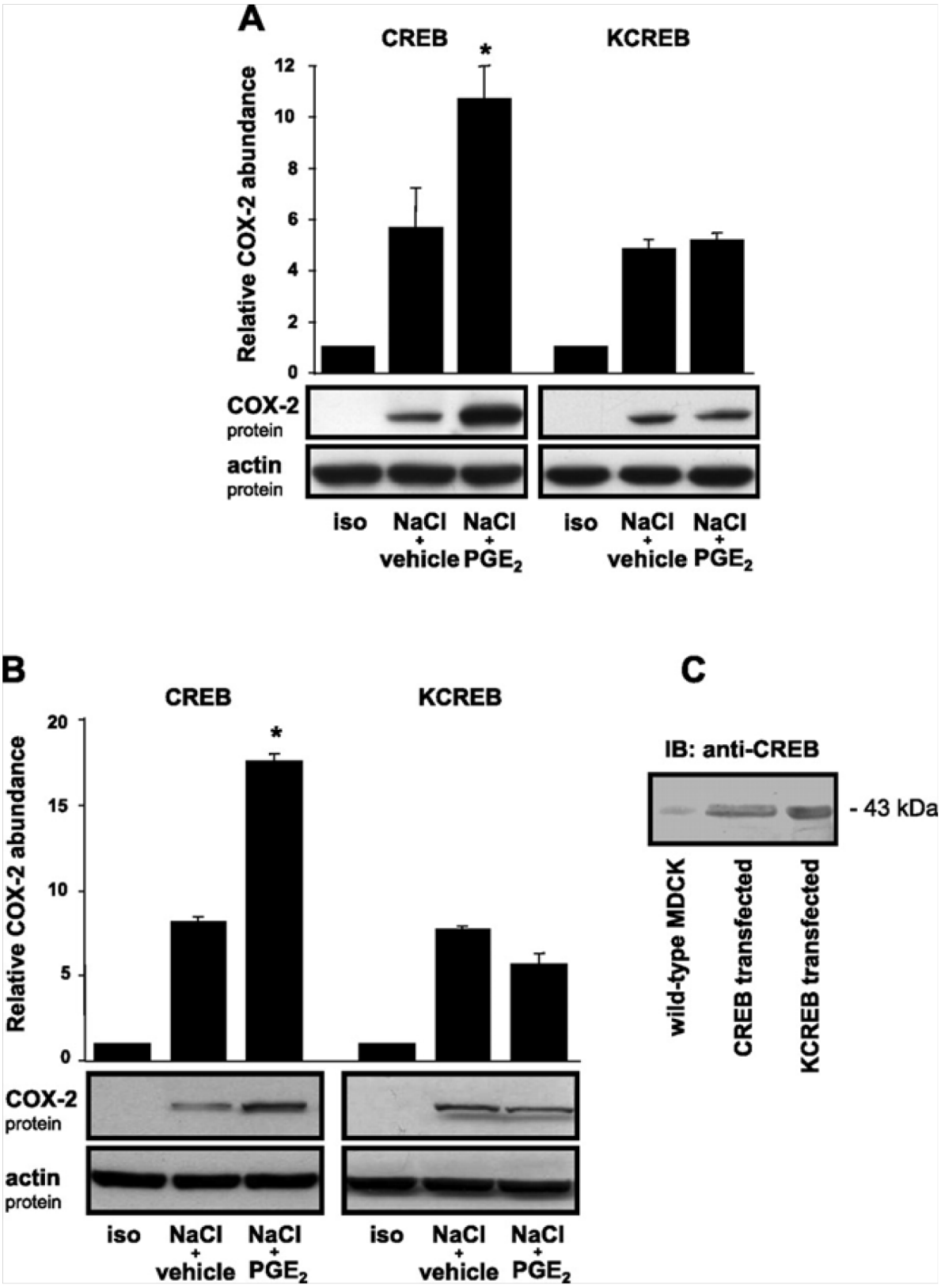
NaCl + PGE2

Figure 8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NaCl + PGE2
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CREB
KCREB
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Relative COX-2 abundance
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protein
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NaCl + vehicle
NaCl + PGE2
iso
NaCl + vehicle
NaCl + PGE2
C
IB: anti-CREB
- 43 kDa
wild-type MDCK
CREB transfected
KCREB transfected

Figure 9

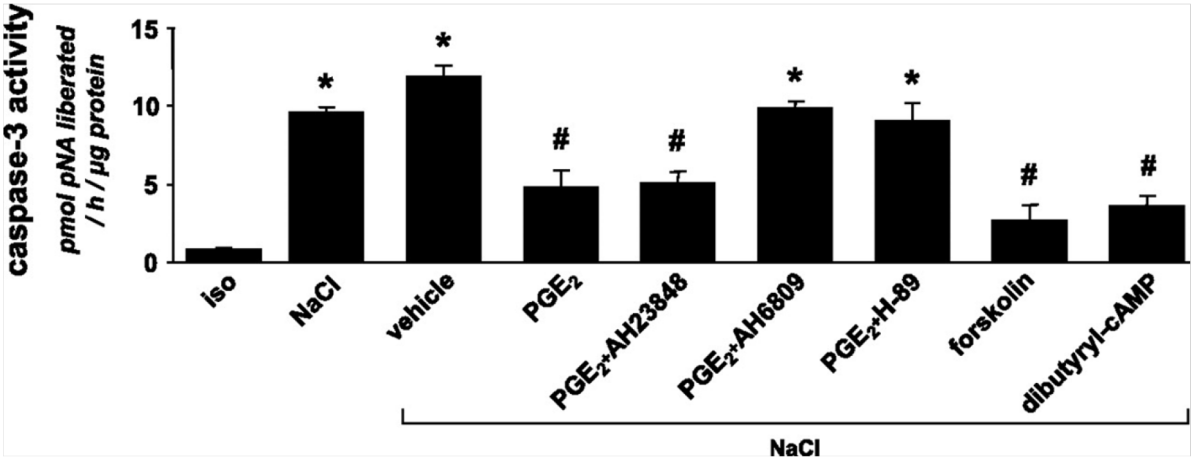
caspase-3 activity
pmol pNA liberated / h / µg protein
15
10
5
0
*
*
*
*
*
#
#
#
#
iso
NaCl
vehicle
PGE2
PGE2+AH23848
PGE2+AH6809
PGE2+H-89
forskolin
dibutyryl-cAMP
NaCl

Figure 10

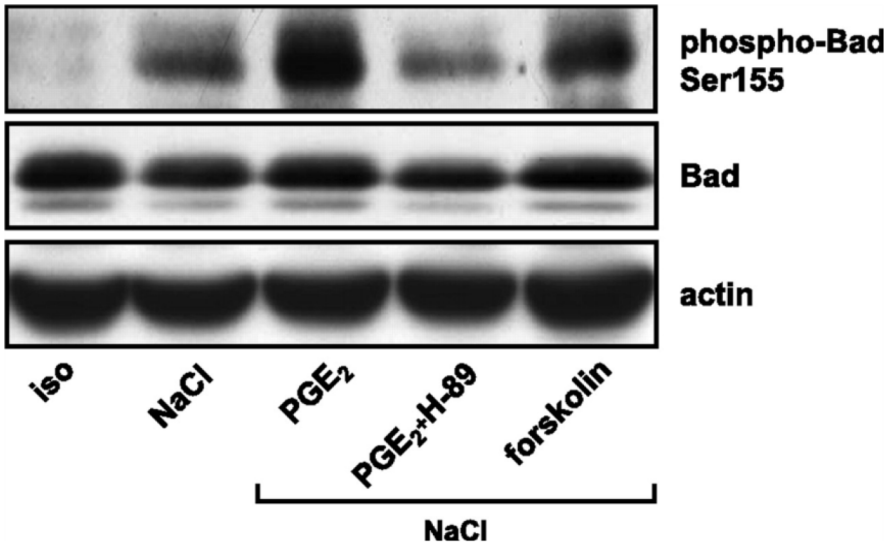
phospho-Bad
Ser155
Bad
actin
iso
NaCl
PGE2
PGE2+H-89
forskolin
NaCl

Figure 11

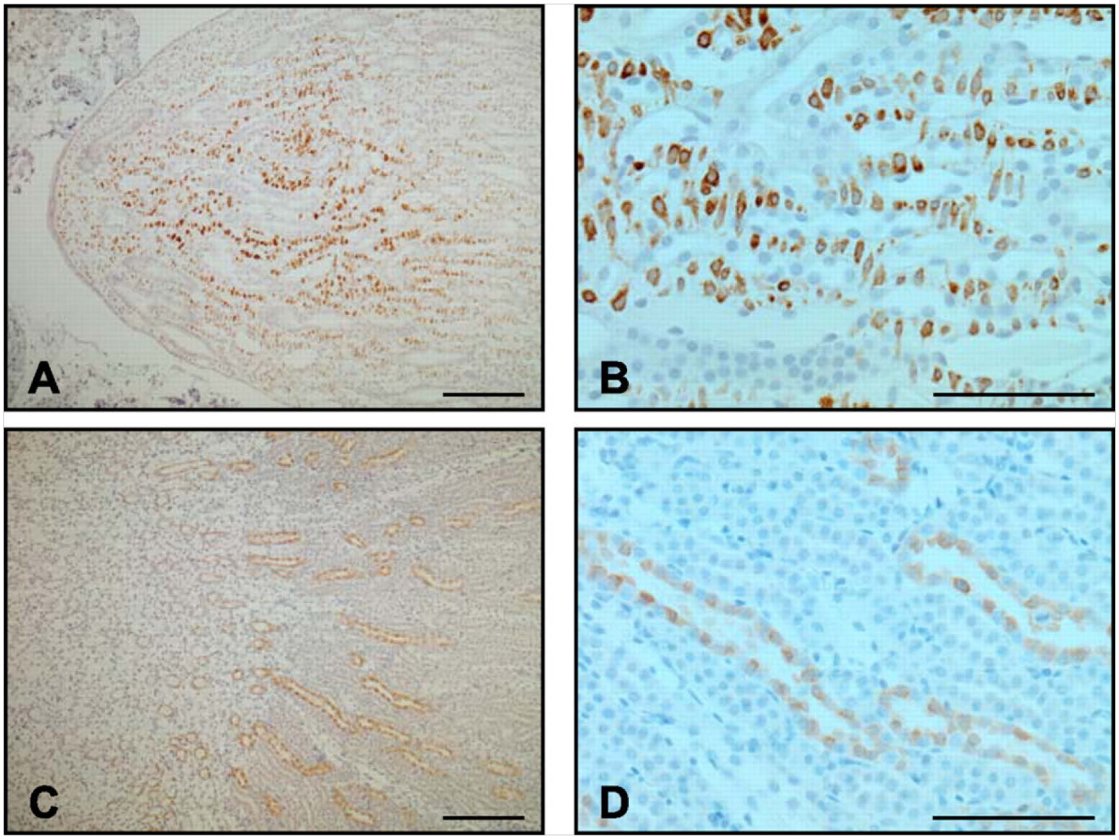

Figure 12

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4. Lebenslauf

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