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Rola kinazy białkowej AMP w regulacji funkcji kłębuszkowej bariery filtracyjnej i cytoszkieletu komórek podocytarnych.

The role of AMP protein kinase in the regulation of glomerular filtration barrier function and podocyte actin cytoskeleton.

Rozprawa na stopień naukowy doktora nauk medycznych
w dyscyplinie biologia medyczna

Promotor: dr hab. inż. Agnieszka Piwkowska, prof. IMDiK PAN

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2. Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowsk M, Angielski S, Piwkowska A, Extracellular ATP modulates podocyte function through P2Y purinergic receptors and pleiotropic effects on AMPK and cAMP/PKA signaling pathways, *Arch Biochem Biophys*, 2020;695:108649. doi: 10.1016/j.abb.2020.108649. Epub 2020 Oct 22., pięcioletni IF 3,480
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Wykaz skrótów używanych w rozprawie doktorskiej

2-MeSATP – *(ang. 2-methylthioadenosine 5'-triphosphate),* 2-metylotioadenozyno-5'-trifosforan

ADP – *(ang. adenosine-5'-diphosphate),* adenozyno-5'-difosforan

AMP – *(ang. adenosine 5'-monophosphate),* adenozyno-5'-monofosforan

AMPK – *(ang. 5'AMP-activated protein kinase),* kinaza białkowa aktywowana przez AMP

ATP - *(ang. adenosine triphosphate)*, adenozyno-5'-trifosforan

ATP-γ-S – *(ang. adenosine 5'-O-(3-thio)triphosphate),* adenozyno-5'-γ-tio-trifosforan

cAMP – *(ang. adenosine 3',5'-cyclic monophosphate),* cykliczny adenozyno-3',5'-monofosforan

cGMP - *(ang. guanosine 3',5'-cyclic monophosphate),* cykliczny guanozyno-3',5'-monofosforan

F-aktyna - *(ang. filamentous actin, F-actin),* aktyna fibrylarna

FITC – *(ang. fluorescein-5-isothiocyanate),* 5-izotiocyjanian fluoresceiny

GTP-aza – *(ang. guanosine-5'triphosphatase),* guanozyno-5'trifosfataza

H-89 - *(ang. (N-[2-p-bromocinnamylamino-ethyl]-5-isoquinolinesulphonamide dihydrochloride),* selektywny inhibitor kinazy białkowej A

HG - *(ang. high glucose),* wysokie stężenie glukozy

MRS2365 - *(ang. (N)-methanocarba-2-methylthioadenosine diphosphate),* selektywny agonista $P2Y_1$

MRS2768 – *(ang. uridine-5'-tetraphosphate δ-phenyl ester tetrasodium salt),* selektywny agonista $P2Y_2$

MRS4062- *(ang. N4-Phenylpropoxycytidine-5'-O-triphosphate tetra(triethylammonium) salt),* selektywny agonista $P2Y_4$

MTF – *(ang. 1,1-Dimethylbiguanide hydrochloride),* metormina

P2X – *(ang. P2X purinoreceptor),* receptory purynowe P2X

P2Y- *(ang. P2Y purinoreceptor),* receptory purynowe P2Y

PKA- *(ang. cAMP- dependent protein kinase),* kinaza białka A

PKGIα- *(ang. cGMP-dependent protein kinase),* kinaza białkowa G typu Iα

Rac1- *(ang. Ras-related C3 botulinum toxin substrate 1),* podobny do Ras substrat 1 składnika C3 toksyny botulinowej

RhoA- *(ang. Ras homolog family member A),* białko A z rodziny homologicznej do Ras

ROS -*(ang. reactive oxygen species),* reaktywne formy tlenu

Rp-8- Br- cAMP – *(ang. 8-Bromoadenosine 3',5'-cyclic Monophosphothioate, Rp-Isomer),* inhibitor kinazy białkowej A, analog cAMP

SG- *(ang. standard glucose),* standardowe stężenie glukozy

TRPC6 -*(ang. Transient Receptor Potential Channel 6),* kanał jonowy receptora potencjału przejściowego typu 6

UDP –*(ang. uridine-5'-diphosphate),* urydyno-5'-difosforan

UTP – *(ang. uridine-5'-triphosphate),* urydyno-5'-trifosforan

VASP - *(ang. vasodilator-stimulated phosphoprotein),* fosfoproteina stymulowane przez czynniki wazodylatacyjne

1. Streszczenie polskojęzyczne i anglojęzyczne

1.1. Streszczenie

Kluczowym elementem kłębuszkowej bariery filtracyjnej są podocyty, które posiadają w swoich wypustkach stopowatych rozbudowany cytoszkielet aktynowy, mający zdolność regulacji wielkości powierzchni filtracyjnej. Wypustki stopowate sąsiednich podocytów tworzą strukturę błony szczelinowej, a kompleks zakotwiczonych w niej białek ściśle kontroluje dynamikę przebudowy cytoszkieletu aktynowego. AMP-zależna kinaza białkowa jest kluczowym enzymem odpowiedzialnym za utrzymanie energetycznej homeostazy oraz właściwą odpowiedź metaboliczną w zależności od zmieniających się warunków środowiskowych, w tym również na warunki stresowe. Podocyty, pokrywające zewnętrzną powierzchnie włośniczek w kłębuszku, wydają się być komórkami szczególnie wrażliwymi na działanie wysokiego stężenia glukozy czy też stresu mechanicznego, co ma miejsce w przebiegu licznych glomerulopatii m.in. nefropatii cukrzycowej. W stanach patofizjologicznych gwałtownie wzrasta uwalniane nukleotydów z komórek podocytarnych, jak i z kłębuszków nerkowych, prowadząc do lokalnego wzrostu ich stężenia w przestrzeni zewnątrzkomórkowej. Uwalniane nukleotydy oraz produkty ich zewnątrzkomórkowej degradacji, działając za pośrednictwem receptorów nukleotydowych P2 pełnią funkcje cząstek sygnałowych regulujących skurcz naczyń, tym samym przyczyniając się do modulacji tempa filtracji kłębuszkowej.

Celem niniejszej pracy było zbadanie wpływu aktywacji kinazy AMP w warunkach hiperglikemii oraz stymulacji receptorów P2 na białka związane z cytoszkieletem aktynowym komórek podocytarnych oraz funkcję kłębuszkowej bariery filtracyjnej.

Doświadczenia przeprowadzono na izolowanych kłębuszkach nerkowych pochodzących od szczurów stada Wistar oraz na szczurzych podocytach hodowli pierwotnej.

W wyniku przeprowadzonych analiz immunoenzymatycznych w podocytach hodowanych w obecności wysokich stężeń glukozy stwierdzono zmniejszenie poziomu fosforylacji AMPK, który ulegał zwiększeniu pod wpływem stymulacji AMPK przez metforminę. Jednocześnie stwierdzono, że obserwowane zmiany są skorelowane z ilością kanału wapniowego TRPC6, stanowiącego istotny elementy błony szczelinowej. W środowisku hiperglikemicznym zaobserwowano wzrost ilości białka TRPC6, która była przywracana do wartości kontrolnych w obecności metforminy. Podobną zależność wykazano w przeprowadzonym barwieniu immunofluorescencyjnym, które wykazało zmniejszenie ilości kompleksu złożonego z TRPC6 oraz podjednostki AMPKα1 w warunkach wysokiego stężenia glukozy oraz zwiększenie ilości tego kompleksu w wyniku działania metforminy. Z przeprowadzonych badań wynika również, że w warunkach wysokiego stężenia glukozy dochodzi do zmniejszenia ilości nefryny, a także zmian w wewnątrzkomórkowej lokalizacji aktyny oraz aktywności białek modulujących cytoszkielet aktynowy. Co więcej, zastosowanie metforminy skutkowało odwróceniem zmian związanych z organizacją włókien aktynowych, dlatego możemy przypuszczać że proces ten jest zależny od AMPK. W przeprowadzonych doświadczeniach na izolowanych kłębuszkach nerkowych i podocytach stwierdziliśmy wzrost przepuszczalności dla albuminy w warunkach hiperglikemii oraz jej znaczny spadek w obecności metforminy, co potwierdza protekcyjne działanie metforminy na kłębuszkową barierę filtracyjną w hiperglikemii.

Przeprowadzone badania wykazały również, że aktywacja receptorów nukleotydowych w komórkach podocytarnych wpływa na równowagę metaboliczną poprzez zwiększenie poziomu fosforylacji AMPK, a także równowagę oksydoredukcyjną poprzez zahamowanie produkcji reaktywnych form tlenu. Spostrzeżono również, że stymulacja purynergiczna wpływa na wielkość syntezy cyklicznych nukleotydów (cAMP i cGMP) regulujących skurcz naczyń krwionośnych. Ponadto, aktywacja receptorów P2, a w szczególności receptora $P2Y_4$,

prowadzi do przebudowy cytoszkieletu aktynowego, czemu towarzyszą spadek aktywności białka RhoA oraz wzrost przepuszczalności dla albuminy przez warstwę podocytów. Proces ten wydaje się być sprzężony z działaniem kinazy białkowej A, której hamowanie aktywności zapobiegało zmianom indukowanym przez stymulację purynergiczną.

Na podstawie otrzymanych danych można wnioskować, że AMP-kinaza, jak i zewnątrzkomórkowe nukleotydy za pośrednictwem receptorów P2 regulują funkcję podocytów, a także modyfikują reorganizację cytoszkieletu aktynowego podocytów oraz przepuszczalność kłębuszkowej bariery filtracyjnej. Obserwowane w badanych warunkach zmiany mogą przyczynić się do lepszego poznania mechanizmów regulujących funkcjonowanie kłębuszkowej bariery filtracyjnej w warunkach fizjologicznych oraz patofizjologicznych.

1.2. Abstract

Podocytes constitute a key element of glomerular filtration barrier mainly through a well-developed contractile apparatus formed by actin and myosin filament bundles. The podocyte foot processes along with the slit diaphragm proteins modulate the actin cytoskeleton dynamics and subsequent glomerular filtration. AMP-activated protein kinase (AMPK) is an essential enzyme responsible for maintaining energy homeostasis and proper metabolic response depending on changing environmental conditions, including stress conditions. Podocytes covering the external surface of the glomerular capillary seem to be a sensitive to a high glucose concentration or mechanical stress during glomerulopathies, including diabetic nephropathy. In pathological conditions release of nucleotides into extracellular space from glomeruli and podocytes is increased. Extracellular nucleotides act as signaling molecules through the P2 nucleotide receptors regulating vasoconstriction, thus contributing to the regulation of the glomerular filtration rate.

The main goal of the study was investigation of the role of AMPK in hyperglycemia and P2 receptors in regulation of podocytes actin cytokeleton and permeability of glomerular filtration barrier.

The experiments were performed using such experimental models as isolated rat glomeruli and primary culture of rat podocytes.

This work demonstrated a decrease of AMPK phosphorylation level in podocytes with high level of glucose and increase of phosphorylation level after AMPK stimulation by metformin. Moreover, we observed that the changes mentioned above are correlated with the amount of TPRC6, which was increased in hyperglycemic conditions and then was restored to control values after AMPK activation. We showed a similar dependence in immunofluorescence staining and the degree of colocalization between AMPK and TRPC6, which was decreased in high glucose concentration and increased by AMPK stimulation. In this study we demonstrated a reduction of the amount of nephrin and changes in intracellular actin distribution in podocytes exposed to high glucose concentrations. Metformin treatment caused restoration of high glucose-induced changes in amount and intracellular location of proteins modulating actin cytoskeleton. Moreover, metformin through AMPK activation caused a reduction of permeability to albumin in podocytes under hyperglycemic conditions and diminution of glomerular permeability in diabetic rats.

We showed that nucleotide stimulation in podocytes has an effect on restoration of energy homeostasis through AMPK activation and maintaining the oxidative balance through decreased ROS generation. Purinergic activation regulates the amount of synthesis of cyclic nucleotides (cGMP and cAMP) controlling the contractility of blood vessels. Furthermore, P2 activation, notably $P2Y_4$, leads to a reorganization of actin cytoskeleton with accompanying suppression of RhoA activity and an increase of permeability to albumin across podocyte

monolayer. The process seems to be coupled with protein kinase A, whose suppression of activity prevented nucleotides-induced changes.

In conclusion, the results of the present study offer evidence supporting a role for AMPK and purinergic signaling in regulating glomerular filtration through podocyte cytoskeleton remodeling. We believe that the proposed studies may become helpful in recognizing and understanding new defense mechanisms on the podocytes functioning, glomerular protection and preservation of normal renal function.

2. Innowacyjność rozprawy

Badania przedstawione w niniejszej dysertacji po raz pierwszy wykazały, że

- aktywacja AMPK przez metforminę reguluje aktywność białek z rodziny Rho oraz moduluje cytoszkielet aktynowy podocytów, wpływając tym samym na przepuszczalność kłębuszkowej bariery filtracyjnej,
- w środowisku hiperglikemicznym ilość białka TRPC6 w podocytach ulega zwiększeniu, natomiast aktywacja AMPK przez metforminę skutkuje zmniejszeniem ilości tego białka,
- aktywacja receptorów P2Y, a w szczególności receptora $P2Y_4$, wpływa na reorganizację cytoszkieletu aktynowego podocytów oraz regulację przepuszczalności przez monowarstwę podocytów poprzez uruchomienie ścieżki sygnałowej cAMP/PKA/RhoA,
- stymulacja receptorów purynergicznych zmniejsza produkcję reaktywnych form tlenu w komórkach podocytarnych.

3. Wstęp

Selektywność procesu filtracji osocza uzależniona jest od budowy filtru kłębuszkowego, który tworzą od strony światła naczynia kapilarnego: komórki śródbłonkowe, błona podstawna oraz komórki nabłonkowe trzewne – podocyty [1]. Wieloletnie badania wykazały, że podocyty odgrywają kluczową rolę w prawidłowym funkcjonowaniu bariery filtracyjnej. O ich unikatowych cechach świadczy chociażby obecność wysoko wyspecjalizowanych struktur komórkowych. Ponadto brak aktywności mitotycznej oraz proliferacyjnej dojrzałych fenotypowo podocytów wskazuje, iż są najbardziej wrażliwym na uszkodzenia elementem kłębuszka nerkowego [2]. W podocytach można wyróżnić trzy odrębne morfologicznie i funkcjonalnie segmenty: unoszące się swobodnie w przestrzeni Bowmanna ciało komórki oraz wypustki główne, a także oplatające zewnętrzną stronę włośniczek wypustki stopowate. Pomiędzy wyrostkami stopowatymi sąsiednich komórek znajdują się wąskie szczeliny filtracyjne, pokryte błoną filtracyjną, która stanowi najbardziej zewnętrzny i zarazem najważniejszy element bariery filtracyjnej [2]. Uszkodzenie struktury szczelin filtracyjnych skutkuje przerwaniem ciągłości i integralności kłębuszkowej bariery filtracyjnej oraz białkomoczem. Charakterystyczne rozlewanie wyrostków stopowatych, które stają się płaskie i szerokie, stanowi cechę wspólną licznych glomerulopatii [3].

W związku z nieustanną ekspozycją na ciśnienie hydrostatyczne filtracji, podocyty przeciwstawiając się siłom sprężystości błony podstawnej regulują wielkość powierzchni filtracyjnej. Adaptacja do warunków środowiskowych uwarunkowana jest obecnością w wypustkach stopowatych elementów kurczliwych takich jak aktyna, aktynina, miozyna, winkulina, wimentyna, paksylina i talina. Ponadto podocyty w wypustkach głównych posiadają rozbudowany cytoszkielet składający się z mikrotubul i filamentów pośrednich, nadający komórce sztywność oraz zapewniający wytrzymałość na rozciąganie i odporność na uszkodzenia mechaniczne [4] [5]. Co więcej, cytoszkielet odgrywa kluczową rolę

w przenoszeniu sygnału generowanego przez siły mechaniczne wymuszające proces filtracji poprzez kompleks białek zakotwiczonych w błonie szczelinowej, jednak dokładny mechanizm regulujący jego dynamikę nie został do końca poznany. Nadrzędną rolę w utrzymaniu struktury błony szczelinowej przypisuje się transbłonowemu białku sygnałowemu – nefrynie, która tworzy szkielet błony szczelinowej na wzór zamka błyskawicznego [6].

Kolejnym ważnym białkiem błony szczelinowej jest TRPC6 (ang. Transient Receptor Potential Channel), który należy do rodziny nieselektywnych kanałów jonowych odpowiedzialnych za napływ jonów wapnia do komórki, aktywowanych w wyniku stymulacji receptorów sprzężonych z białkiem G lub receptorów kinazy tyrozynowej [7]. Z ostatnio opublikowanych doniesień naukowych wynika, że kluczowe znaczenie w patogenezie nefropatii cukrzycowej może mieć zwiększenie ekspresji kanału wapniowego TRPC6, u podłoża którego leży nadmierna aktywność angiotensyny II (ANG II) oraz nasilenie stresu oksydacyjnego [8]. Wykazano, że nadekspresja TRPC6 prowadzi do wzrostu wewnątrzkomórkowego stężenia wapnia, co powoduje zahamowanie ekspresji synaptopodyny i nefryny oraz prowadzi do dezorganizacji F-aktyny, zniszczenia wyrostków stopowatych, zwiększonej filtracji kłębuszkowej i następczej proteinurii [9]. Powyższe zmiany skorelowane są ze stymulacją aktywności białka RhoA, które obok Rac1 i Cdc42 należy do najlepiej poznanych białek z rodziny GTP-az Rho, pełniących rolę przełączników komórkowych w kontrolowaniu wielu ścieżek sygnałowych m.in. związanych z reorganizacją cytoszkieletu aktynowego. Liczne eksperymenty wykazały istnienie ścisłej zależności między zmianami w aktywności białek Rho a zaburzeniami w strukturze wyrostków stopowatych oraz w konsekwencji białkomoczem [10] [11].

Kinaza białkowa aktywowana przez AMP (AMPK) jest enzymem, który odgrywa kluczową rolę w ochronie funkcji komórek w czasie niedoboru energetycznego. AMPK, aktywowana

w odpowiedzi na wzrastające stężenie wewnątrzkomórkowego AMP, bierze udział w regulacji komórkowego bilansu energetycznego. Jej zadaniem jest przełączenie komórek ze stanu anabolicznego do katabolicznego, poprzez zamykanie szlaków zużywających ATP i uruchomianie procesów generujących energię. AMPK wykazuje wielokierunkowe działanie, w wyniku jej aktywacji zahamowane zostają procesy syntezy glukozy, tłuszczów, białek oraz wzrostu komórek, a pobudzane są wychwyt i oksydacja kwasów tłuszczowych oraz wychwyt glukozy wraz z glikolizą [12]. Ze względu na pełnione przez AMPK funkcje, w stanach związanych z insulinoopornością takich jak otyłość, zespół metaboliczny czy cukrzyca obserwuje się obniżenie aktywności AMPK [13]. Wyniki badań molekularnych przeprowadzonych w naszym zespole dowiodły, że wskutek długotrwałej ekspozycji komórek podocytarnych na wysokie stężenie glukozy (HG), w komórkach tych dochodzi do indukcji insulinooporności, przejawiającej się zniesieniem stymulującego efektu insuliny na dokomórkowy transport glukozy w sposób zależny od AMPK [14].

W procesach związanych z uszkodzeniem komórki oraz stanie zapalnym wzrastające stężenie ATP w płynie zewnątrzkomórkowym [15] w wyniku długotrwałej ekspozycji zwiększa również ilość wolnych rodników tlenowych [16]. Zwiększona produkcja ROS jest czynnikiem, który nasila progresję przewlekłej choroby nerek (PChN), a u pacjentów stwierdza się podwyższoną aktywność markerów stresu oksydacyjnego [17]. Badania ostatnich lat dostarczyły również wiedzy na temat roli kinazy AMP w regulacji równowagi oksydoredukcyjnej [13]. W poprzednich pracach wykazaliśmy, że w warunkach hiperglikemii metformina aktywując AMPK, działa jednocześnie antyoksydacyjnie poprzez zmniejszenie aktywności oksydazy NADPH (głównego źródła reaktywnych form tlenu w tych komórkach) [18] [19]. Ponadto efekt ten jest związany z zahamowaniem aktywności enzymów degradujących ATP (ekto-ATPaz), skutkującym wzrostem stężenia zewnątrzkomórkowego ATP oraz aktywacją receptorów nukleotydowych P2. Na podstawie różnic w budowie

i sposobie przekazywania sygnału, wyodrębniono dwie podgrupy tych receptorów: P2X i P2Y. Receptory P2X, które obejmują 7 podtypów ($P2X_{1-7}$), są błonowymi kanałami jonowymi dla kationów Na^+, K^+ oraz Ca^{2+}, których otwarcie w odpowiedzi na zewnątrzkomórkowe ATP powoduje przepływ jonów zgodnie z gradientem elektrochemicznym. Z kolei, receptory P2Y, pośród których wyróżniamy 8 podtypów ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11-14}$), aktywowane są przez nukleotydy adeninowe (ATP, ADP) oraz urydynowe (UTP, UDP). P2Y należą do receptorów metabotropowych, sprzężonych z trójpodjednostkowymi białkami G. W zależności od rodzaju białka G z jakim są związane, przekazują sygnał za pośrednictwem białek efektorowych: cyklazy adenylowej, fosfolipazy C, uwalniając przy tym cząsteczki przekaźników II-rzędu jak cykliczny AMP (cAMP), diacyloglicerol (DAG), kationy wapnia (Ca^{2+}), trisfosforan inozytolu (IP_3) [20].

Uwalnianie ATP oraz innych nukleotydów do przestrzeni pozakomórkowej zarówno przez izolowane kłębuszki nerkowe, jak również podocyty zachodzi w sposób konstytutywny. W wyniku drażnienia mechanicznego, zapalenia czy uszkodzenia błony komórkowej ulega ono intensyfikacji [21]. Pozakomórkowy ATP wpływa na motorykę naczyń kapilarnych kłębuszka nerkowego. Za pośrednictwem receptorów P2X i P2Y wywołuje on skurcz bądź relaksację kłębuszków, regulując tym samym wielkość powierzchni filtracyjnej [22]. Ponadto, poznane zostało rozkurczające działanie zewnątrzkomórkowego ATP na kłębuszki nerkowe na drodze syntezy i uwolnienia tlenku azotu i następczej aktywacji cyklazy guanylanowej, wzrostu stężenia cGMP i stymulacji kinazy białkowej G typu I α (PKGIα) [23]. Wykazano, że substancje wazodylatacyjne, które wpływają na zmiany stężenia cyklicznych nukleotydów (cAMP i cGMP) biorą tym samym udział w regulacji napięcia mięśni gładkich naczyń krwionośnych oraz mikrokrążenia, a także powodują przegrupowanie włókien F-aktyny w komórkach podocytarnych [24]. Z kolei wzrost stężenia cAMP może efektywnie zwiększać fosforylację AMPK na drodze aktywacji cAMP-zależnej kinazy białkowej (PKA) [25]. Biorąc

pod uwagę powyższe informacje, wydaje się prawdopodobne, że kinaza AMP zajmuje centralne miejsce w regulacji dynamiki cytoszkieletu aktynowego podocytów. Mimo rosnącego zainteresowania badaniami nad AMPK, wciąż niewiele wiadomo na temat roli tego enzymu w fizjologii oraz patofizjologii nerek. Obserwacje kliniczne wykazały, że metformina, obecnie lek referencyjny w farmakoterapii cukrzycy typu 2, poza skutecznością w wyrównaniu gospodarki węglowodanowej oraz lipidowej ma również działanie nefroprotekcyjne manifestujące się obniżeniem mikroalbuminurii u pacjentów z cukrzycą typu 2 [26]. Postuluje się, że wspomniane wyżej zmiany są ściśle związane z aktywacją AMPK [27]. Mimo to, brakuje danych literaturowych dotyczących dokładnego mechanizmu działania oraz ścieżek sygnałowych regulujących wpływ AMPK na przepuszczalność filtru kłębuszkowego.

4. Cele pracy

Cel główny

Celem obecnej pracy było zbadanie roli kinazy AMP w regulacji przepuszczalności kłębuszkowej bariery filtracyjnej.

Cele szczegółowe

- Próba scharakteryzowania wewnątrzkomórkowych ścieżek sygnałowych sprzężonych z kinazą AMP, leżących u podstaw zmian dynamiki cytoszkieletu aktynowego komórek podocytarnych.
- Ocena zależności między aktywnością kinazy AMP a ilością kanału wapniowego TRPC6 w podocytach.
- Ocena wpływu aktywności AMPK w warunkach hiperglikemii na białka związane z cytoszkieletem aktynowym komórek podocytarnych, a także funkcję kłębuszkowej bariery filtracyjnej.
- Identyfikacja wewnątrzkomórkowych mechanizmów wpływających na dynamikę cytoszkieletu aktynowego oraz funkcję komórek podocytarnych w warunkach farmakologicznej aktywacji receptorów P2Y z uwzględnieniem potencjalnej roli kinaz białkowych: AMPK, PKA oraz PKG.
- Ocena wpływu farmakologicznej modulacji receptorów P2Y na wewnątrzkomórkowe stężenie cyklicznych nukleotydów cAMP oraz cGMP.

5. Materiały i metody

Doświadczenia przeprowadzono na szczurach stada Wistar (samice 100-120 g i samce c.c. 200-250 g) pochodzących z hodowli Instytutu Medycyny Doświadczalnej i Klinicznej im. Mirosława Mossakowskiego Polskiej Akademii Nauk w Warszawie. Wszystkie procedury badawcze wykonano zgodnie z Dyrektywa Parlamentu Europejskiego i Rady 2010/63/UE z dnia 22 września 2010 r. w sprawie ochrony zwierząt wykorzystywanych do celów naukowych oraz za zgodą Lokalnej Komisji Etycznej w Bydgoszczy.

5.1. Hodowla szczurzych podocytów

Pierwszym modelem badawczym była hodowla pierwotna szczurzych podocytów, którą prowadzono w oparciu o standardową procedurę opracowaną w Instytucie Anatomii i Biologii Komórkowej Uniwersytetu w Heidelbergu (Niemcy)[28][29]. Kłębuszki nerkowe izolowano techniką przesiewową przez sita metalowe o średnicy oczek 160, 106 oraz 53 µm. Otrzymaną zawiesinę komórkową zawieszano w pożywce hodowlanej, którą rozdzielano na butelki hodowlane i umieszczano na 6 dni w inkubatorze (37◦C, 5% CO_2 i 95% powietrze). Po 6 dniach inkubacji przyklejone kłębuszki nerkowe wraz z pozostałymi komórkami poddawano trypsynizacji, a otrzymaną zawiesinę przesiewano przez sito nylonowe o średnicy oczek 33 µm. Otrzymany przesącz zawierający podocyty wysiewano na naczynia hodowlane, a następnie umieszczano w inkubatorze (37◦C, 5% CO_2 i 95% powietrze). Eksperymenty na komórkach podocytarnych zostały przeprowadzone między 12 a 20 dniem hodowli. Fenotyp hodowanych komórek badano metodą immunocytochemiczną przy użyciu przeciwciał skierowanych przeciwko swoistym białkom podocytów: nefryny, podocyny oraz podokaliksyny. Doświadczenia prowadzono w trzech układach badawczych, w których podocyty inkubowane były w następujących warunkach:

- 5-cio dniowa inkubacja w środowisku o standardowym stężenia glukozy (11,1 mM D-glukoza) oraz wysokim stężeniu glukozy (30 mM D-glukoza) w obecności

aktywatora (metformina, MTF= 2mM) lub inhibitora (związek C, CC= 100μM) kinazy AMP

- 1 min inkubacja z agonistami receptorów P2: ATP (100 μM) oraz ATP-γ-S, 2MeS-ATP oraz selektywnymi agonistami receptorów $P2Y_1$ (MRS2365), $P2Y_2$ (MRS2768) oraz $P2Y_4$ (MRS4062) w stężeniu 10 μM.
- 1 h preinkubacja z inhibitorami kinazy A: H89 oraz Rp-8-Br-cAMP (1h, 10 μM) oraz 1 min inkubacja z ATP (100 μM) oraz MRS4062 (10 μM).
- transfekcja komórek podocytarnych wyciszającym RNA (siRNA) przeciwko AMPKα1 oraz AMPKα2 zgodnie z protokołem załączonym przez producenta (Santa Cruz Biotechnology).

Po określonym czasie inkubacji w poszczególnych układach badawczych, komórki poddawano odpowiedniej lizie, a następnie wykonano analizy:

1. Immunodetekcja białek metodą Western Blot.

   Rozdział elektroforetyczny mieszaniny białek (20 μg) przeprowadzano w 10 % żelu poliakrylamidowym, którą następnie przenoszono w polu elektrycznym na membrany PVDF przy użyciu transferu półsuchego. Dokonano detekcji białek metodą kolorymetryczną z użyciem fosfatazy alkaicznej.

2. Ocena ekspresji genowej badanych białek przy użyciu metody real-time PCR
3. Ocena wewnątrzkomórkowej lokalizacji białek za pomocą barwienia immunofluorescencyjnego, uzyskany obraz analizowano pod mikroskopem konfokalnym Nikon z użyciem oprogramowania NIS-Elements lub LeicaSP8X.
4. Ocena aktywności białek Rac1 oraz RhoA metodą immunoenzymatyczną przy użyciu zestawów biochemicznych RhoA i Rac1 G-LISA® Activation Assay Biochem Kit™ (Cytoskeleton Inc., USA).

5. Oznaczanie stężenia cGMP oraz cAMP metodą immunoenzymatyczną (ELISA) przy użyciu komercyjnie dostępnych zestawów (Cyclic GMP/cyclic AMP Competitive ELISA Kit, Thermo Fischer Scientific) w lizatach komórek podocytarnych.
6. Oznaczenie poziomu reaktywnych form tlenu przy użyciu sondy fluorescencyjnej DCF (2,7-dichlorofluoresceina).
7. Pomiar przepuszczalności dla albuminy znakowanej fluoresceiną (FITC-albumina) przez warstwę podocytów utworzoną na miękkiej nylonowej błonie, pokrytej kolagenem typu IV [30].

5.2. Izolowane kłębuszki nerkowe

Drugim modelem badawczym były kłębuszki nerkowe izolowane ze szczurów zdrowych (K) i szczurów z indukowaną farmakologicznie cukrzycą (STZ), którym podawano dootrzewnowo streptozotocynę (65 mg kg m.c.). Kłębuszki nerkowe izolowano z samców techniką przesiewową przez sita o średnicy oczek 250, 125 oraz 75 μm [31].

- Na izolowanych kłębuszkach nerkowych zawieszonych w roztworze PBS zawierającym 5% BSA badano przepuszczalność filtru kłębuszkowego dla albuminy na podstawie zmiany objętości izolowanych kłębków spowodowanej gradientem ciśnienia onkotycznego pomiędzy środowiskiem inkubacyjnym a przestrzenią wewnątrz kapilarna kłębuszków nerkowych.
- W homogenatach tkankowych oceniano ilość badanych białek metodą Western blot.
- W moczu oznaczano stężenie albuminy metodą immunoenzymatyczną przy użyciu kitu biochemicznego AssayMax Rat Albumin ELISA kit (Assaypro, St. Charles, MO, USA).
- We krwi oceniano stężenie glukozy metodą oksydazową przy użyciu glukometru (Accu-chek Go, Roche Diagnostics GmbH).

- Oceniano klirens radioaktywnej inuliny oraz przepływ krwi przez korę nerki metodą laser-Doppler.
- Oceniano dobowe spożycie wody oraz wydalanie moczu.

Wyniki z przeprowadzonych doświadczeń przedstawiono jako wartość średnią ± standardowy błąd średniej (SEM) z n = liczba powtórzeń. Różnice statystyczne pomiędzy wynikami oceniano przy zastosowaniu jednoczynnikowej analizy wariancji (one-way ANOVA) przy użyciu programu SigmaPlot 11.0 (Systat Software, Inc., USA) oraz GraphPad Prism 6. Za statystycznie znamienną różnicę między wynikami uznawano wartość $P<0,05$.

6. Omówienie wyników

Przewlekła hiperglikemia oraz towarzysząca jej insulinooporność prowadzą do zaburzeń aktywności szlaków zaangażowanych w regulację homeostazy glukozy, do których niewątpliwie należy ścieżka regulowana przez AMP-zależną kinazę białkową. W pierwszym etapie badań wykazaliśmy, że długotrwała ekspozycja szczurzych podocytów na wysokie stężenie glukozy powodowała zmniejszenie poziomu fosforylacji AMPK (Publikacja 1, Fig 2B), natomiast aktywacja AMPK przez metforminę skutkowała wzrostem poziomu fosforylacji w środowisku o standardowym (SG, 11 mM) oraz wysokim stężeniu glukozy (HG, 30 mM).

Wpływ aktywności AMPK na ilość białka kanału wapniowego TRPC6 w szczurzych podocytach

Kolejnym zadaniem badawczym była ocena wpływu wysokiego stężenia glukozy na poziom ekspresji białka oraz ilość kanału wapniowego TRPC6 oraz zależności pomiędzy AMPK a TRPC6 w szczurzych podocytach w warunkach hiperglikemicznych. Otrzymane wyniki wykazały, że w środowisku o wysokim stężeniu glukozy wzrasta zarówno ekspresja genowa, jak i ilość białka TRPC6 (Publikacja 1., Fig. 1A, B). Co więcej, stymulacja AMPK przez metforminę w HG powoduje przywrócenie ilości TRPC6 do wartości obserwowanych w warunkach kontrolnych (Publikacja 1, Fig. 2A). Doświadczenia z zastosowaniem wyciszającego RNA wobec AMPK wskazały jednoznacznie na udział podjednostki α1 w regulacji ekspresji TRPC6. Zahamowanie ekspresji genu AMPKα1 powodowało zwiększenie ilości kanału wapniowego TRPC6, która była przywracana do wartości kontrolnych w obecności metforminy (Publikacja 1, Fig. 3B). Otrzymane wyniki znalazły potwierdzenie w przeprowadzonym podwójnym barwieniu immunofluorescencyjnym oraz analizie kolokalizacji, które wykazały zmniejszenie ilości kompleksu złożonego z TRPC6 oraz podjednostki AMPKα1 w warunkach wysokiego stężenia glukozy oraz zwiększenie

w wyniku działania metforminy (Publikacja 1, Fig. 4). Otrzymane wyniki sugerują, że AMPK bierze udział w TRPC6-zależnej regulacji homeostazy jonów wapnia w podocytach. Ponadto farmakologiczna aktywacja AMPK w warunkach wysokiego stężenia glukozy może kompensować wzrost stężenia wewnątrzkomórkowego Ca^{2+}.

Wpływ kinazy AMP na regulację ścieżek sygnałowych związanych z cytoszkieletem aktynowym podocytów

W kolejnych doświadczeniach postanowiliśmy zbadać udział AMPK w regulacji białek kontrolujących stan organizacji aktyny w podocytach. W środowisku hiperglikemicznym, zarówno w komórkach podocytarnych, jak i kłębuszkach nerkowych obserwowaliśmy zmniejszenie ilości nefryny (Publikacja 1, Fig. 6A, B). Ponadto w podocytach wskutek działania metforminy następowało zniesienie tego efektu (Publikacja 1, Fig. 6A). Otrzymane wyniki skorelowane były z obrazem immunofluorescencyjnym, gdzie spostrzeżono zanik sygnału pochodzącego od nefryny w wypustkach stopowatych w podocytach w HG oraz nasilenie jego intensywności w obrębie ciała komórki pod wpływem metforminy (Publikacja1, Fig. 6C). Nasze badania pokazały również, że w warunkach hiperglikemii zmniejsza się stopień fosforylacji Rac1, który ulega zwiększeniu po zastosowaniu metforminy (Publikacja 1, Fig. 7A). Podobną zależność dla białka Rac1 wykazała analiza aktywności enzymatycznej, która malała pod wpływem HG i znacznie wzrastała w wyniku działania MTF (Publikacja 1, Fig. 7B). Ponadto wykazano zwiększenie aktywności enzymatycznej białka RhoA w środowisku hiperglikemicznym oraz zmniejszenie pod wpływem metforminy (Publikacja 1, Fig. 7C). Przeprowadzone barwienie immunofluorescencyjne wykazało też zmiany w wewnątrzkomórkowej lokalizacji aktyny w badanym układzie. W środowisku HG sygnał pochodzący od wybarwionej fluorescencyjnie falloidyną aktyny układał się w wiązki o charakterze pofragmentowanym w porównaniu do SG, gdzie miał charakter ciągły, rozłożony równomiernie. W komórkach inkubowanych w warunkach hiperglikemii z aktywatorem

AMPK obraz immunofluorescencyjny dla aktyny był zbliżony do obserwowanego w warunkach kontrolnych (Publikacja 1, Fig. 9).

Wpływ kinazy AMP na regulację przepuszczalności kłębuszkowej bariery filtracyjnej

Reorganizacja cytoszkieletu aktynowego jak i modyfikacje w aktywności białek wpływających na jego układ przestrzenny w analizowanych warunkach zostały potwierdzone zmianami przepuszczalności dla albuminy. W środowisku hiperglikemicznym zaobserwowaliśmy znaczny wzrost przepuszczalności dla albuminy, zarówno przez monowarstwę podocytów jak i izolowane kłębuszki nerkowe. W wyniku działania metforminy obserwowaliśmy zmniejszenie przepuszczalności dla albuminy (Publikacja 1, Fig. 10 A,B). Wydaje się prawdopodobne, że w warunkach hiperglikemii zmiany w ekspresji nefryny jak i białek regulujących dynamikę organizacji włókien aktyny, skutkujące destabilizacją kompleksu białek błony szczelinowej oraz utratą szczelności filtru kłębuszkowego, są skorelowane z osłabieniem funkcji AMPK. Natomiast farmakologiczna aktywacja tejże kinazy może zasadniczo wpływać na przywrócenie morfologii oraz prawidłowego funkcjonowania bariery filtracyjnej

Wpływ aktywacji receptorów purynergicznych na poziom fosforylacji AMPK

Kinaza AMP jako główny regulator obrotu energetycznego w komórce, wrażliwa na zmiany wewnątrzkomórkowego stężenia nukleotydów, wydaje się być ważnym ogniwem w zewnątrzkomórkowej sygnalizacji nukleotydowej. Na kolejnym etapie badań podjęto próbę zidentyfikowania roli AMPK w kaskadzie sygnalizacyjnej aktywowanej przez receptory P2. W pierwszej kolejności scharakteryzowano dynamikę zmian w poziomie fosforylacji AMPK w obecności zewnątrzkomórkowego ATP w zależności od czasu. Otrzymane wyniki pokazały, że w 1. minucie inkubacji z zewnątrzkomórkowym ATP następuje gwałtowny wzrost poziomu fosforylacji AMPK, który spada wraz z upływem czasu, powracając w 10 minucie do wartości kontrolnych (Publikacja 2, Fig. 1A). Zatem otrzymane wyniki sugerują,

że kinaza AMP odgrywa istotną rolę w przywróceniu homeostazy energetycznej, a najsilniejszy efekt obserwowany jest już w 1. minucie.

Zgodnie z danymi literaturowymi opisującymi występowanie receptorów P2Y w podocytach [32][33], do kolejnych eksperymentów wybrano nieselektywnych agonistów receptorów P2 (ATP, 2-Me-S-ATP oraz ATP-γ-S) oraz specyficznych dla receptorów $P2Y_1$, $P2Y_2$ oraz $P2Y_4$. W obecności zastosowanych związków obserwowano wzrost poziomu fosforylacji AMPK, a statystycznie znamienne różnice odnotowano wobec ATP, ATP-γ-S oraz 2-MeS-ATP oraz agonisty dla receptora $P2Y_4$ (MRS4062) (Publikacja 2, Fig. 2B).

Wpływ aktywacji purynergicznej na produkcję ROS

Ostatnie doniesienia literaturowe dowodzą , że AMPK jest nie tylko czuła na zmiany stężenia nukleotydów, ale również ROS [34]. W świetle przedstawionych informacji, oceniano poziom ROS pod wpływem stymulacji purynergicznej, który ulegał obniżeniu pod wpływem ATP oraz agonistów dla receptorów $P2Y_1$ (MRS2365) oraz $P2Y_4$ (MRS4062) (Publikacja 2, Fig. 1D). Otrzymane wyniki sugerują zatem, że krótkotrwała stymulacja receptorów nukleotydowych P2Y aktywuje kinazę AMP oraz zmniejsza produkcję ROS w podocytach. Zatem wydaje się prawdopodobne, że w warunkach zachwiania bilansu nukleotydowego w podocytach jednym z głównych zadań AMPK jest przywrócenie nie tylko stanu równowagi energetycznej, ale również oksydoredukcyjnej.

Wpływ aktywacji purynergicznej na stężenie cAMP oraz cGMP

Dotychczasowe badania wskazują na udział receptorów P2Y w przekaźnictwie sygnału wapniowego w komórkach podocytarnych [32] [35], jednakże brakuje informacji odnośnie aktywowania innych ścieżek sygnałowych. Na podstawie przeprowadzonych doświadczeń z użyciem zewnątrzkomórkowego ATP wykazaliśmy wzrost stężenia cGMP oraz cAMP. Z kolei zastosowanie selektywnych agonistów P2Y skutkowało spadkiem stężenia cGMP oraz

wzrostem cAMP, a w przypadku agonisty receptora $P2Y_4$ obserwowane zmiany miały charakter statystycznie znamienny (Publikacja 2, Fig 2A, B). Uzyskane rezultaty mogą sugerować, że aktywacja receptorów P2Y w podocytach uruchamia procesy zależne od cAMP, czego potwierdzeniem był również wzrost poziomu fosforylacji białka VASP w miejscu Ser157, które to jest kontrolowane przez PKA (Publikacja 2, Fig. 2C).

Wpływ aktywacji purynergicznej na białka związane z cytoszkieletem aktynowym

W kolejnych doświadczeniach postanowiliśmy sprawdzić czy nukleotydowa stymulacja może wpływać na cytoszkielet komórek podocytarnych poprzez regulację aktywności białka RhoA. Nasze badania wykazały spadek aktywności białka RhoA w środowisku inkubacyjnym z dodatkiem ATP, ATP-γ-S oraz agonisty receptora $P2Y_4$ (Publikacja 2, Fig. 3A). Powyższe wyniki sugerują, że aktywacja receptorów nukleotydowych może promować rozkurcz aparatu kurczliwego podocytów, o czym również świadczy spadek poziomu fosforylacji łańcuchów lekkich miozyny w analizowanych warunkach (Publikacja 2, Fig. 3B).

Wpływ aktywacji purynergicznej na wzajemne działanie AMPK oraz PKA w regulacji białek związanych z cytoszkieletem aktynowym podocytów

Na tym etapie wysunęliśmy hipotezę badawczą, że aktywacja purynergiczna, a w szczególności aktywacja receptora $P2Y_4$, odpowiada ze transdukcję sygnału do cytoszkieletu komórki poprzez oddziaływanie na szlak związany z białkiem RhoA. Ponadto założyliśmy, że powyższy mechanizm jest regulowany przez sprzężone działanie kinaz AMPK oraz PKA. W związku z powyższym, kolejne doświadczenia z użyciem ATP oraz MRS4062 przeprowadzono w warunkach hamujących aktywność PKA z zastosowaniem dwóch inhibitorów: H-89 oraz Rp-8-Br-cAMP. W zastosowanych warunkach doświadczalnych nie zaobserwowano, aby działanie AMPK było regulowane przez PKA. Preinkubacja komórek z inhibitorami PKA nie eliminowała indukowanego przez P2Y

wzrostu poziomu fosforylacji AMPK (Publikacja 2, Fig. 4A, B). Zaobserwowano natomiast, że zahamowanie aktywności PKA zapobiegało zmianom aktywności białka RhoA stymulowanym przez agonistów receptorów nukleotydowych. (Publikacja 2, Fig. 6A, B). Na podstawie analizy immunofluorescencyjnej stwierdzono również zwiększenie intensywności sygnału dla PKA w obszarach przybłonowych pod wpływem ATP oraz MRS4062. Ponadto zahamowanie aktywności kinazy A zwiększało translokację białka RhoA do błony komórkowej, która była eliminowana w obecności ATP oraz MRS4062 (Publikacja 2, Fig. 7). Inkubacja komórek podocytarnych z ATP oraz $P2Y_4$ powodowała również zmiany w wewnątrzkomórkowej dystrybucji F-aktyny w podocytach polegającej na zwiększeniu intensywności immunofluorescencyjnej izotiocyjanianu falloidyny w obszarach przybłonowych wraz z towarzyszącym zmniejszeniem intensywności fluorescencyjnej w obszarach okołojądrowych. Z kolei zmniejszenie aktywności PKA zapobiegało reorganizacji włókien F-aktyny (Publikacja 2, Fig. 9).

Wpływ aktywacji purynergicznej na przepuszczalność monowarstwy podocytów dla albuminy

Na podstawie przeprowadzonych eksperymentów w warunkach farmakologicznej aktywacji receptorów nukleotydowych stwierdziliśmy wzrost przepuszczalności przez monowarstwę podocytów dla albuminy. W warunkach doświadczalnych z zastosowaniem inhibitorów kinazy białkowej A obserwowano redukcję przepuszczalności do wartości obserwowanych w warunkach kontrolnych (Publikacja 2, Fig. 10). Na podstawie otrzymanych danych można wnioskować, że główną ścieżką sygnałową uruchamianą w odpowiedzi na stymulację nukleotydową w podocytach jest szlak zależny od cAMP/PKA, który reguluje dynamikę cytoszkieletu aktynowego podocytów oraz przepuszczalność bariery filtracyjnej.

7. Podsumowanie wyników

Wyniki badań zawarte w niniejszej rozprawie sugerują, iż AMP-zależna kinaza białkowa może być istotnym ogniwem w regulacji przepuszczalności kłębuszkowej bariery filtracyjnej. Otrzymane przez nas wyniki wspierają tezę o protekcyjnym działaniu AMPK na komórki podocytarne. Przeprowadzone doświadczenia pokazały, że AMPK przywraca ilość kanału wapniowego TRPC6, a także wpływa na przebudowę cytoszkieletu aktynowego w podocytach w warunkach hiperglikemii. Ponadto uruchomienie natychmiastowej kaskady sygnalizacyjnej z udziałem AMPK w odpowiedzi na nukleotydową stymulację podkreśla jej niezwykle ważną funkcję w utrzymaniu równowagi energetycznej. Skutecznym sposobem ochrony komórek przed działaniem stresu oksydacyjnego wydaje się być powiązanie aktywacji AMPK z obniżeniem produkcji ROS. Co więcej, przedstawione dane eksperymentalne wskazują na współudział PKA w nukleotydowej modyfikacji cytoszkieletu aktynowego oraz przepuszczalności monowarstwy podocytów dla albuminy.

8. Wnioski

- AMP-zależna kinaza białkowa reguluje homeostazę wapnia w podocytach poprzez modyfikację ilości kanału wapniowego TRPC6.

- AMP-zależna kinaza białkowa bierze udział w reorganizacji cytoszkieletu aktynowego komórek podocytarnych i tym samym wpływa na regulację przepuszczalności kłębuszkowej bariery filtracyjnej.

- Wydaje się prawdopodobne, że aktywacja AMPK przez metforminę może odwracać zmiany w organizacji włókien aktyny powodowane przez hiperglikemię oraz wpływać na położenie i kształt wypustek stopowatych podocytów, a tym samym wielkość powierzchni filtracyjnej.

- Zewnątrzkomórkowe ATP za pośrednictwem receptorów P2 reguluje funkcję podocytów poprzez plejotropowe działanie zależne od cAMP/PKA oraz AMPK.

- Wydaje się prawdopodobne, że wśród receptorów P2Y największy udział w reorganizacji cytoszkieletu aktynowego w komórkach podocytarnych może mieć receptor $P2Y_4$.

- Farmakologiczna aktywacja receptorów P2Y poprzez zmiany stężenia cyklicznych nukleotydów (cAMP, cGMP) jest jednym z mechanizmów regulujących organizację cytoszkieletu komórkowego w podocytach.

- Zewnątrzkomórkowe nukleotydy kontrolują przebudowę cytoszkieletu komórkowego oraz procesy związane z ruchem komórek podocytarnych, wpływając tym samym na wielkość powierzchni filtracyjnej.

- Uruchomienie kaskady sygnalizacyjnej z udziałem AMPK może mieć istotne znaczenie w przywróceniu równowagi energetycznej oraz utrzymaniu równowagi oksydacyjnej.

- Powyższe obserwacje mogą być użyteczne w wyjaśnieniu podłoża molekularnego patogenezy zaburzeń czynnościowych bariery filtracyjnej, a także stać się pomocne w świetle obecnych poszukiwań nowych strategii diagnostycznych i terapeutycznych w leczeniu glomerulopatii.

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Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions

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ABSTRACT

Podocytes have foot processes that comprise an important cellular layer of the glomerular barrier involved in regulating glomerular permeability. The disturbance of podocyte function plays a central role in the development of proteinuria in diabetic nephropathy. AMP-activated protein kinase (AMPK), a key regulator of glucose and fatty acid metabolism, plays a major role in obesity and type 2 diabetes. Accumulating evidence suggests that TRPC6 channels are crucial mediators of calcium transport in podocytes, and these channels are involved in disturbing the glomerular filtration barrier in diabetes.

Metformin is an anti-diabetic drug widely used for treating patients with type 2 diabetes. Recent studies have suggested that the therapeutic effect of metformin might be mediated by AMPK. The precise function of metformin on cellular function and intracellular signaling in podocytes under diabetic conditions is not fully understood.

In this study, we demonstrated that metformin normalized TRPC6 expression via AMPKα1 activation in podocytes exposed to high glucose concentrations. A quantitative analysis showed that metformin increased the colocalization of TRPC6 and AMPKα1 subunits from 42% to 61% in standard glucose (SG) medium and from 29% to 52% in high glucose (HG) medium. AMPK activation was also necessary for maintaining appropriate levels of Rho-family small GTPase activity in HG conditions. Moreover, metformin through AMPK activation remodeled cytoskeleton dynamics, and consequently, reduced filtration barrier permeability in diabetic conditions.

1. Introduction

Central to the development of type 2 diabetes is the impairment of insulin action in skeletal muscle, fat tissue, and liver. The resulting dysregulation of glucose and fat metabolism leads to kidney failure and/or cardiovascular complications. Thus, type 2 diabetes can be debilitating or even fatal. The majority of patients with albuminuria and end-stage renal failure in the Western world have abnormalities in insulin production or insulin effectiveness [1,2].

Podocytes are highly specialized cells that wrap around glomerular capillaries, and they comprise a key component of the glomerular filtration barrier. Podocytes consist of three morphologically and functionally different segments: a cell body, major processes, and foot processes. The podocyte cell body extends primary processes that branch into foot processes; the foot processes of neighboring podocytes interact to establish a highly branched, interdigitating pattern, known as the slit diaphragm [3]. The slit diaphragm represents a signaling platform with many proteins that regulate podocyte function, including nephrin, podocin, Neph1, CD2AP, TRPC6, BK_{Ca}, and actin [4–6].

Podocytes are the target of injury in many glomerular diseases, including arterial hypertension and diabetes mellitus. Studies in patients with microalbuminuric type 1 diabetes found that podocyte foot processes in patients with diabetes were wider than those in healthy patients. In fact, foot process width was correlated directly with the urinary albumin excretion rate [7]. In addition, the number and density of podocytes were reported to be markedly reduced in patients with either type 1 or type 2 diabetes compared to controls [8,9]. These data suggested that podocyte injury is an important feature of diabetic kidney disease.

The mammalian homologues of Drosophila canonical transient receptor potential channels (TRPCs) are potent plasma membrane channels that contribute to changes in the cytosolic free Ca^{2+} concentration. They either act as Ca^{2+} entry pathways in the plasma membrane or they modulate the membrane-driving force for Ca^{2+} entry by changing the membrane. Recently, mutations in the TRPC6 gene have been

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linked to the human proteinuric kidney disease, focal segmental glomerulosclerosis (FSGS) [10]. In this disease, podocyte foot processes (slit diaphragm) lose integrity in their specific cellular junction structure, which disrupts the glomerular filtration barrier. A previous study showed that overexpression of TRPC6 in the mouse kidney resulted in the induction of proteinuria [11]. However, it remains unclear how the channel activity of mutated TRPC6 is involved in the pathogenesis of FSGS. Another study showed that the P112Q mutation in TRPC6 increased its plasma membrane expression [12], which suggested that changes in surface expression might contribute to the pathogenesis of the disease. Moreover, another study found an important link between Ca^{2+} and podocyte injury; they showed that the activation of the Ca^{2+}-dependent phosphatase, calcineurin, led to synaptopodin cleavage and proteinuria [10]. Moreover, overexpressing TRPC6 in podocytes could lead to higher intracellular Ca^{2+} concentrations, in the presence of stimuli. An increase in intracellular Ca^{2+} could downregulate the expression of nephrin and synaptopodin and stimulate RhoA activity, which in turn would cause the derangement of F-actin and a reduction in foot processes [13]. Additionally, in cultured podocytes, sustained TRPC6 activation induced apoptosis [14]. In analogy to other cell types, such as neuronal dendritic spines, it is reasonable to assume that the dynamic responses of podocytes and their foot processes might be mediated by cytoskeletal elements and Ca^{2+}-dependent processes. It is probable that TRPC6 is normally part of a compensatory response to mechanical or metabolic stress in podocytes; thus, inhibiting these channels might actually be counterproductive in glomerular diseases.

Metformin is an anti-diabetic drug widely used to treat patients with type 2 diabetes [15]. The proposed mechanisms of metformin action include a reduction in hepatic gluconeogenesis and, to a lesser extent, an increase in glucose uptake into skeletal muscle cells. Recent studies have suggested that the therapeutic effect of metformin might be mediated by AMP-activated kinase (AMPK) [15–17]. It was also demonstrated that metformin reduced albuminuria in type 2 diabetes mellitus [18,19]. The precise function of metformin in cellular function and intracellular signaling in podocytes under diabetic conditions is not fully understood. In this study, we demonstrated that metformin normalized TRPC6 channel expression via AMPKα1 activation in podocytes exposed to high glucose concentrations. Moreover, metformin through AMPK activation and regulation of Rho-family small GTPase activity remodeled cytoskeleton dynamics, and consequently, reduced filtration barrier permeability. These findings suggest that the protective effect of metformin on podocyte cytoskeleton involves AMPK-dependent decrease of TRPC6 channel expression, which ameliorates podocyte function in hyperglycemia.

2. Methods

2.1. Preparation and culture of rat podocytes

All experimental procedures were performed in accordance with directive 2010/63/EU and were approved by the local Bioethics Commission in Bydgoszcz. We used female Wistar rats that weighed 100–120 g. Podocytes were isolated as described previously [20]. Experiments were conducted with podocytes cultivated for 12–20 days. Cell phenotypes were established with podocyte-specific antibodies against Wilms tumor-1 protein (WT-1; Biotrend Koeln, Germany) and nephrin (ProSci Inc., Poway, Ca, USA). For the different experiments, cells were cultured in standard glucose (SG, 11.1 mM) and high glucose (HG, 30 mM) media for five days. AMPK activity in podocytes was modulated by exposure to an activator (metformin, 2 mM, 5 days) or inhibitor (compound C, 0.1 mM, 2 h).

2.2. Experimental animals and metabolic balance studies

Studies were performed with male diabetic rats (streptozotocin 65 mg/kg, i.p.) and age-matched control Wistar rats (weight: 200–240 g) obtained from Mossakowski Medical Research Center, Polish Academy of Sciences, Warsaw (Poland). Experiments were performed after 12 days in animals with blood glucose concentrations > 17 mM. Rats were housed singly in metabolic cages for 24 h with free access to a regular pellet diet and drinking water. Urine was collected, and diuresis was measured. Urinary albumin excretion was measured with the AssayMax Rat Albumin ELISA kit (Assaypro, St. Charles, MO, USA). After an overnight fast, blood was collected from decapitated animals to determine baseline serum glucose and insulin values. Glucose concentrations were measured in the whole blood samples with a glucose oxidase method (Accu-chek Go, Roche Diagnostics GmbH) (Table 1).

Table 1
Metabolic balance studies in control and diabetic Wistar rats.

Parameter	Control rats n = 10	Diabetic rats n = 12
Body weight, g	240 ± 10	231 ± 12
Water intake, ml/24 h	26 ± 2	119 ± 12*
Urine volume, ml/24 h	9.9 ± 2.3	89.6 ± 9.2*
Blood glucose concentration, mg/dl	103 ± 8	378 ± 30*
Urinary albumin excretion, μg/24 h	90.2 ± 12	193 ± 29*

Values are the means ± SE.
* P < 0.05 vs. control rats.

2.3. Permeability assay

Transepithelial permeability to albumin was evaluated by measuring the diffusion of FITC-labeled BSA (Sigma) across a podocyte monolayer, as described by Oshima et al., with minor modifications [21,22]. Briefly, rat podocytes (1×105 cells/cm^2) were seeded on type IV collagen-coated Cell Culture Inserts (3-μm membrane pore size, 0.32 cm^2 membrane surface area, BD Biosciences). Inserts were placed in 24-well plates, and the cells were allowed to differentiate for one week. Cells were used for experiments between post-seeding days 7 and 15. Before use in experiments, the podocytes were washed twice with PBS and medium on both sides of the insert, and the medium was replaced with serum-free RPMI 1640 medium (SFM). After 2 h, the medium in the upper compartment was replaced with 0.3 ml fresh SFM, and that in the lower compartment was replaced with 1.5 ml SFM containing 1 mg/ml FITC-albumin. After 1 h incubation, 200 μl of the solution in the upper chamber was transferred to a 96-well plate, and the content of FITC-albumin was determined by measuring absorbance at 490 nm with a plate spectrophotometer (BioTek EL808).

2.4. Glomerular permeability to albumin in vitro

Glomeruli were isolated from male diabetic rats (streptozotocin 65 mg/kg, i.p.) and age-matched control Wistar rats (weight: 200–240 g). The volume response of glomerular capillaries to an oncotic gradient generated by defined concentrations of albumin was measured as described previously [20]. Isolated glomeruli (de-capsulated and devoid of afferent and efferent arterioles [23]) were allowed to affix to glass coverslips coated with poly-L-lysine (1 mg/ml). They were then incubated in medium containing 5% BSA and either AMPK inhibitor (compound C, 100 μM, 20 min) or AMPK activator (metformin, 2 mM, 30 min) at 37 °C. Next, the compounds were washed out with 5% BSA medium. The initial incubation medium was replaced with medium containing 1% BSA to generate an oncotic gradient across the glomerular capillary wall. Control glomeruli were treated with equivalent volumes of buffer containing 5% BSA (i.e., which did not generate an oncotic gradient). The glomerular volume responses were recorded with videomicroscopy (Olympus microscope IX51) before and 1 min after the test reagents were added. Glomerular volume (V) was calculated based on the surface area (S) of the glomerulus with the formula: $V = 3/4\pi\ (S/\pi)3/4$ with cellSens Dimension software

(Olympus). We found a direct relationship between the increase in glomerular volume (ΔV), calculated as (Vfinal − Vinitial) / Vinitial, and the oncotic gradient (ΔΠ) applied across the capillary wall. This principle was used to calculate the reflection coefficient of albumin (σalb), defined as the ratio of ΔVs measured in the presence (experimental) and absence (control) of an oncotic gradient:

$$\sigma alb = \Delta V experimental / \Delta V control$$

The reflection coefficient of albumin was then used to calculate the glomerular capillary permeability to albumin (convectional Palb = 1 − σalb), which described the movement of albumin consequent to water flow. At least ten glomeruli isolated from three or more rats were studied in each experiment.

2.5. Laser Doppler renal flowmetry

Studies were performed in male diabetic (streptozotocin 65 mg/kg, i.p.) and age-matched control Wistar rats (weight: 200–250 g). Experiments were performed after 12 days in animals with blood glucose concentrations > 17 mM (Accu-Check Go, Roche, Basel, Switzerland). Procedures were approved by the local Bioethics Commission in Bydgoszcz.

Polyethylene catheters were inserted into the femoral vein and carotid artery (PE-50) and into the abdominal aorta near the renal arteries (PE-10), accessed from the femoral artery of anesthetized (Inactin, 100 mg/kg, i.p.) tracheostomized rats. The urinary bladder and left ureter were catheterized. The animals received intravenous infusions of 150 mM NaCl (bolus 200 μl, then sustained perfusion at 45 μl/min) containing 5 μCi/ml [^{3}H]-inulin. The left kidney was exposed and placed in a Lucite holder. Blood flow to the superficial renal cortex (CBF) was measured with the laser-Doppler method with a Periflux 4001 system (Perimed AB, Jarfalla, Sweden); the CBF probe was placed on the kidney surface. After surgery recovery, the first three 10-min collection periods were used as controls. Then, metformin (50 mg/kg body weight) was injected into the abdominal aorta, and urine was collected in three, 10-min periods. The CBF was continuously monitored (experimental period). Upon completion of the experiment, all the animals were killed with an anesthesia overdose, and the tip of the aortic catheter was checked to ensure that it had been properly positioned near the renal arteries.

2.6. Western blot analysis

To obtain podocyte and glomerular lysates, the cells were treated with lysis buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol), in the presence of a protease inhibitor cocktail, and homogenized at 4 °C by scraping. Proteins (15 μg) were separated on a 10% SDS-polyacrylamide gel and electrotransferred to nitrocellulose membranes. The following primary antibodies were used for Western blotting: anti-p-AMPKα (Thr172) (1:1000, Cell Signaling Technology), anti-AMPKα (1:1000, Cell Signaling Technology), anti-AMPKα1 (1:500, Santa Cruz Biotechnology), anti-AMPKα2 (1:500, Santa Cruz Biotechnology), anti-nephrin (1:1000, Sigma-Aldrich), anti-TRPC6 (1:1000, Sigma-Aldrich), anti-p-PAK 1/2/3 (Thr 423/402/421) (1:800, Sigma-Aldrich), anti-PAK1/2/3 (1:800, Cell Signaling Technology), anti-ROCK1 (1:1000, Cell Signaling Technology), anti-ROCK2 (1:1000, Cell Signaling Technology), anti-p-cofilin (1:1000, Sigma-Aldrich), anti-cofilin (1:1000, Santa Cruz Biotechnology), anti-p-Rac1 (Ser71) (1:1000, OriGene), and anti-actin (1:3000, Sigma-Aldrich). To detect the primary antibodies, the membranes were incubated with the appropriate alkaline phosphatase-labeled secondary antibodies. The protein bands were visualized with the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium (BCIP/NBT) system.

2.7. RNA interference and cell transfection

Podocytes were transfected with small interfering RNAs (siRNAs) that targeted AMPKα1 and AMPKα2 (Santa Cruz Biotechnology). Controls were transfected with non-silencing siRNA (scrambled siRNA, negative control) (Santa Cruz Biotechnology). Cells were cultured in RPMI 1640 supplemented with 10% FBS. One day before the experiment, the culture medium was changed to antibiotic-free RPMI 1640 supplemented with 10% FBS. The cells were transfected with the siRNA Transfection Reagent (Santa Cruz Biotechnology), according to the manufacturer's instructions. Briefly, the targeted siRNAs (or scrambled siRNA) were diluted in Transfection Medium (final concentration, 80 nM), mixed with siRNA Transfection Reagent, and incubated for 30 min at room temperature. Then, Transfection Medium was added to the transfection mixture, mixed gently, and added to the podocytes. After 7 h, we added growth medium supplemented with 2× concentrations of FBS and antibiotics. The podocytes were incubated for an additional 24 h. After transfection, gene silencing was checked at the protein level with Western blotting.

2.8. Immunofluorescence

Podocytes were seeded onto coverslips coated with type 1 collagen (Becton Dickinson Labware, Becton, UK) and cultured in RPMI 1640 supplemented with 10% FBS. Cells were fixed in PBS plus 4% formaldehyde for 10 min at room temperature. Fixed podocytes were permeabilized with 0.3% Triton-X for 3–4 min, then blocked with PBSB solution (PBS plus 2% FBS, 2% BSA, and 0.2% fish gelatin) for 1 h. After blocking, cells were incubated with anti-cofilin (1:50), anti-TRPC6 (1:100), anti-AMPKα1 (1:100), and anti-AMPKα2 (1:100) antibodies in PBSB at 4 °C for 1 h. Then, cells were incubated with blocking peptide to eliminate non-specific staining. Next, the cells were washed three times with cold PBS and incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:1000) or Alexa Fluor 546 (1:1000). Actin was stained with Alexa Fluor 594 phalloidin (1:200). Specimens were imaged with a confocal laser scanning microscope (Leica SP8X) equipped with a 63× oil immersion lens.

2.9. RNA extraction and real-time PCR analysis

From cultured podocytes, total RNA was isolated with TRI Reagent (Sigma) and chloroform/isopropanol extraction. The quantity of RNA was determined with spectrometry, and purity was assessed by calculating A260/A280. A RNA was considered pure when the A260/A280 ratio was 1.8–2.2. Next, RNA was treated with DNase (Sigma), and reverse transcription was performed with 700 ng RNA and 100 U M-MLV Reverse Transcriptase (Promega) in a mixture of M-MLV Reaction Buffer, 0.2 mM dNTPs, 10 mM DTT, 0.25 μg Primer p(dT)$_{15}$ (Roche), and 8 U RNAse Inhibitor (EURx).

The mRNA levels of ATP2A1, ATP2A2, ATP2A3, ATP2B1, ATP2B3, ATP2B4, and TRPC6 were determined with TaqMan hydrolysis probes (Roche) and gene-specific intron-spanning primers in a real-time PCR (RT-PCR) assay performed on a Light Cycler 480 (Roche). The results were quantified with the ΔΔCt method, where β-actin served as an internal control. The RT-PCR conditions included pre-incubation for 10 min at 95 °C; then 45 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 60 °C, and elongation for 1 s at 72 °C. Negative controls included dH_2O instead of cDNA template. Amplified products were electrophoresed in a 2.5% agarose gel, visualized with ethidium bromide, and imaged with the GelDoc-It Imaging System (UVP, Cambridge, UK). Table 2 shows the primers utilized.

2.10. Rac1 and RhoA activity assays

Rac1 and RhoA activation were measurement in the supernatant with a commercially available G-LISA Rac1 Activation Assay Biochem Kit (BK128; Cytoskeleton, Inc.) and a G-LISA RhoA Activation Assay Biochem Kit (BK124; Cytoskeleton, Inc.).

Table 2
Real-time PCR primers.

Gene name	GenBank accession number	Primer sequences (5′–3′)	Product size
ATP2A1	NM_058213.1	F: TCGGAACTATCTGGAGGGATAA R: GGATCTGTGACACGGTTCAA	63 bp
ATP2A2	NM_001110139.2	F: CAGTCCTAACTGTGGTGTTTTCC R: TTAGGAAGCGGTTACTCCAGTATT	78 bp
ATP2A3	NM_012914.1	F: AGGCATCACGTGGATGAAA R: GAGGGCCGAACTGGAGAC	73 bp
ATP2B1	NM_053311.1	F: CTGCTCAGAACTTTCAATAGAACAA R: GGTTGGAATGGTTGAAATAAGC	94 bp
ATP2B3	NM_133288.1	F: TGGACCAGCCCTCAAAAA R: ATGTCAGAGGCCTCCTTGG	81 bp
ATP2B4	NM_001005871.1	F: CTCTGAAAATCGCAACAAAGC R: AGTGGCTGGATTTCCAAGG	60 bp
TRPC6	NM_053559.1	F: CAGCCGTTTAAAACTCGCTATT R: ACCACGAGGAATTTCACTGC	141 bp
β-Actin	NM_031144.3	F: CTAAGGCCAACCGTGAAAAG R: GCCTGGATGGCTACGTACA	79 bp

2.11. Statistical analysis

Statistical analyses were performed with one-way ANOVAs, followed by the Student-Newman-Keuls test to determine significance. Values are reported as means ± SEMs. Significance was set at $P < 0.05$.

3. Results

3.1. Diabetes influence on Ca^{2+}-ATPase and TRPC6 expression in podocytes and glomeruli

We performed RT-PCR with total RNA extracted from primary cultured rat podocytes to evaluate mRNA expression of the three isoforms of sarco/endoplasmic reticulum Ca^{2+}-ATPases: ATP2A1, ATP2A2, and ATP2A3; the three isoforms of plasma membrane Ca^{2+}-ATPases: ATP2B1, ATP2B3, and ATP2B4; and the TRPC6 channel. We found that the ATP2B2 isoform was not expressed in podocytes [24]. We also investigated the influence of high glucose (HG) concentrations on the expression of mRNA isoforms (Fig. 1A). A prolonged incubation in HG (30 mM, 5 days) increased mRNA expression of all the genes we investigated, except genes that encoded the ATP2A3 and ATP2B4 isoforms. Moreover, we observed increase of TRPC6 channel protein level in podocytes about 90% (Fig. 1B) and in isolated glomeruli about 60% (Fig. 1C) under diabetic conditions.

3.2. The role of AMPK activity in TRPC6 channel expression

To evaluate the effect of AMPK on TRPC6 protein levels in podocytes, cells were incubated in SG or HG medium in the presence of Metformin (AMPK activator, 2 mM) or Compound C (AMPK inhibitor, 100 μM). We demonstrated that HG reduced AMPK phosphorylation in podocytes and glomeruli. Moreover, in glomeruli isolated from diabetic rats, we observed reductions in the expression of total AMPKα, AMPKα1, and AMPKα2 by 26%, 20%, and 35%, respectively (Fig. 2). When podocytes exposed to HG were treated with metformin, AMPK activation increased and TRPC6 channel expression was restored to the level observed in SG medium (Fig. 2A). Therefore, we postulated that AMPK played an essential role in the regulation of TRPC6 channel expression in podocytes.

We then evaluated the roles of the AMPKα1 and AMPKα2 subunits in the regulation of TRPC6 expression. We used siRNAs to knock down AMPKα1 and AMPKα2 expression by 40% and 49%, respectively (Fig. 3A). In controls, the downregulation of AMPKα1 expression induced an 82% increase in TRPC6 protein levels (from 0.654 ± 0.124 to 1.191 ± 0.032, $P < 0.05$, Fig. 3B) and a 34% decrease in AMPKα phosphorylation (from 1.002 ± 0.061 to 0.645 ± 0.090, $P < 0.05$, Fig. 3C). Metformin treatment restored TRPC6 protein levels and AMPK phosphorylation levels to values observed in controls transfected with scrambled siRNA.

Moreover, immunofluorescence experiments showed that metformin caused substantial changes in the subcellular localization of AMPKα1 subunits in cultured rat podocytes (Fig. 4). Metformin increased the intensity of AMPKα1 immunostaining close to the cell surface in SG and HG medium. However, we did not observe any change in AMPKα2 localization (Fig. 5). A quantitative analysis showed that metformin increased the colocalization of TRPC6 and AMPKα1 subunits from 42% to 61% in SG medium and from 29% to 52% in HG medium ($n = 8–10$, $P < 0.05$, Fig. 4). However, metformin did not influence the level of TRPC6 that colocalized with AMPKα2 subunits (Fig. 5).

3.3. The role of AMPK activity on nephrin expression and localization in podocytes

Nephrin is required for the regulation of podocyte function. It has been shown that nephrin was critical for stabilizing podocyte cytoarchitecture and for insulin action [25,26].

We found that diabetic conditions reduced nephrin expression (Fig. 6A,B). We hypothesized that this reduction might be due to impaired AMPK-dependent regulation of the actin cytoskeleton. We modified AMPK activity with the AMPK activator, metformin (2 mM), and the AMPK inhibitor, compound C (100 μM) (Fig. 6A). In podocytes exposed to HG medium, we found that metformin increased nephrin expression by 34% (0.786 ± 0.070 vs. control 0.586 ± 0.053, $P < 0.05$, Fig. 6A). Conversely, in podocytes grown in SG medium, the AMPK inhibitor, compound C, decreased nephrin protein levels to values observed in HG medium. Moreover, immunofluorescence experiments showed that HG exposure caused substantial changes in the subcellular localization of nephrin; in HG medium, nephrin expression was reduced in podocyte processes. Metformin treatment increased the amount of nephrin detected close to the cell surface (Fig. 6C).

3.4. The role of AMPK in HG-dependent regulation of Rho-family small GTPases in podocytes

The function of podocytes is closely associated with the actin cytoskeleton. Rho-family small GTPases play a crucial role in cytoskeletal architecture and in regulating cell migration and growth [27]. To evaluate the effect of AMPK activation on small GTPases (Rac1 and RhoA) in podocytes, cells were incubated in SG and HG medium in the presence of metformin or compound C. We found that HG medium induced a 24% reduction in Rac1 serine 71 (Ser71)- phosphorylation and a 20% reduction in Rac1 activity, compared to controls (Fig. 7A,B).

Metformin treatment increased Rac1 Ser71-phosphorylation by 26% (1.54 ± 0.07 vs. control 1.22 ± 0.08, $n = 6$, $P < 0.05$) in SG

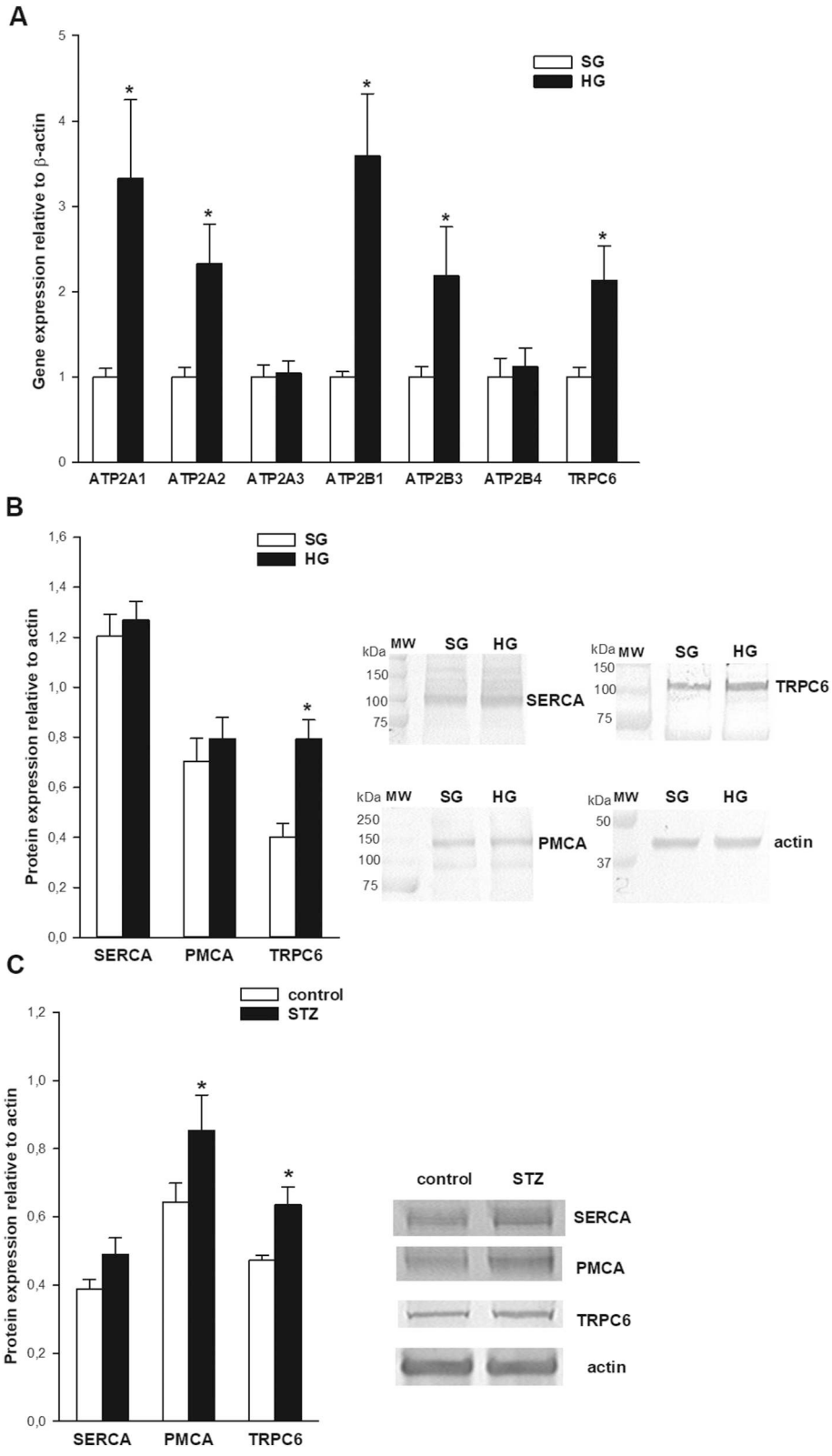

Fig. 1. The influence of diabetic conditions on SERCA, PMCA, and TRPC6 expression. Rat podocytes were cultured in standard glucose (SG, 11 mM) and high glucose (HG, 30 mM) concentrations for five days. Glomeruli were isolated from control and diabetic (STZ) Wistar rats. (A) Real-time PCR results show ATP2a, ATP2b, TRPC6, and β-actin gene expression profiles. (B,C) SERCA, PMCA, and TRPC6 protein expression levels were analyzed in (B) podocytes and (C) isolated glomeruli. Proteins (20 μg) were separated with SDS-PAGE, immunoblotted with anti-SERCA, anti-PMCA, anti-TRPC6, and anti-actin antibodies, and visualized with an alkaline phosphatase-reaction. Values are the means ± SEM (n = 4–6). *P < 0.05 compared to control. Abbreviations: SERCA: sarco/endoplasmic reticulum Ca2+-ATPases; PMCA: plasma membrane Ca2+-ATPases.

medium and by 35% (1.26 ± 0.16 vs. control 0.93 ± 0.04, n = 6, P < 0.05, Fig. 7A) in HG medium. Moreover, metformin also induced increases in Rac1 activity by 39% in SG (1.21 ± 0.14 vs. control 0.87 ± 0.07, n = 5, P < 0.05) and by 64% in HG (1.18 ± 0.13 vs. control 0.72 ± 0.09, n = 5, P < 0.05, Fig. 7B). Inhibiting AMPK activity with compound C reduced Rac1-GTP binding and Rac1 phosphorylation to the values observed in HG medium (Fig. 7A,B). We also found that HG medium increased RhoA activity (from 0.175 ± 0.006 to 0.281 ± 0.017, n = 5, P < 0.05, Fig. 7C). The same effect was observed in the presence of compound C, in both SG and HG conditions. Conversely, metformin treatment resulted in a reduction in RhoA activity in HG to the value observed in SG conditions (Fig. 7C). The ROCK1 and ROCK2 protein levels were not altered in

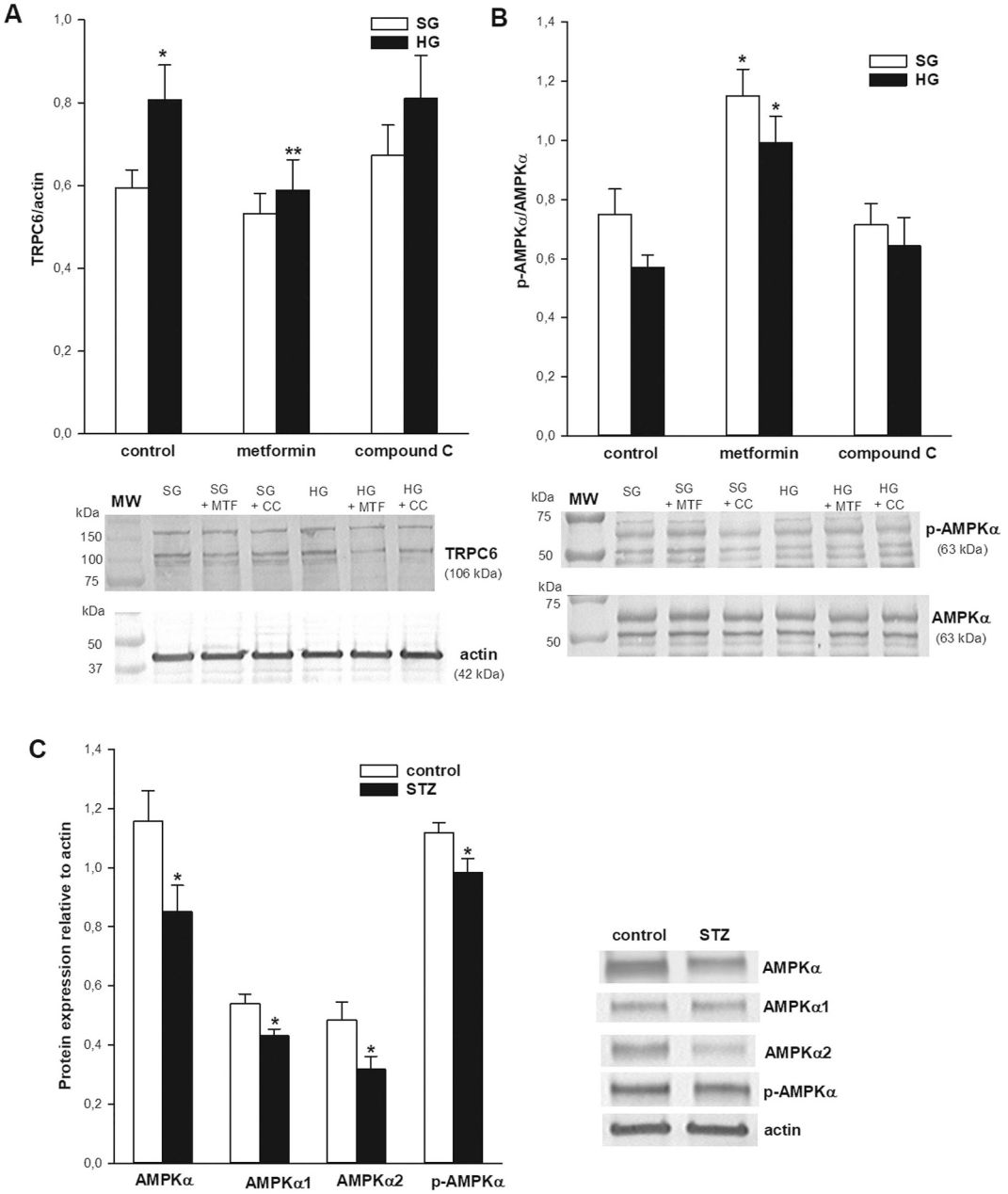

Fig. 2. The influence of AMPK activity on TRPC6 channel expression. Podocytes were cultured in the presence of standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) concentrations and treated with metformin or compound C. We performed densitometric quantification of podocyte lysates to determine (A) the amount of TRPC6 protein and (B) the degree of AMPK phosphorylation. The lanes correspond to the following treatment conditions: (1) SG, (2) SG + metformin, (3) SG + compound C, (4) HG, (5) HG + metformin, (6) HG + compound C. (C) Glomeruli were isolated from control and diabetic (STZ) Wistar rats. Diabetic conditions caused reductions in the amounts of AMPKα, AMPKα1, and AMPKα2 proteins and AMPKα phosphorylation. Actin was used as a loading control. Values are the mean ± SEM (n = 4–6). *P < 0.05 vs. SG, **P < 0.05 vs. HG.

podocytes or isolated glomeruli under diabetic conditions (Fig. 7D,E,F). These data suggested that AMPK activation was necessary for maintaining appropriate levels of Rho-family small GTPase activity in HG conditions.

3.5. AMPK activity regulates actin cytoskeleton remodeling in podocytes

The dynamics of actin filament assembly/disassembly and its organization in podocytes are regulated by several actin-binding proteins, including ADF/cofilins [28]. In addition, p21-activated kinase (PAK) signals to cofilin in response to insulin, which facilitates both cortical actin remodeling and glucose uptake in skeletal muscle cells [29]. Notably, cofilin is activated when it is dephosphorylated. Accordingly, we examined the effects of AMPK on PAK and cofilin activities in podocytes. Cells were incubated in HG medium and compared to cells cultured in SG medium in the presence of metformin or compound C. HG reduced PAK phosphorylation from 0.552 ± 0.051 to 0.432 ± 0.034 (P < 0.05, Fig. 8A). Metformin increased PAK phosphorylation in SG-cultured cells by 43% (0.788 ± 0.080 vs. control 0.552 ± 0.051, P < 0.05) and restored PAK phosphorylation in podocytes cultured in HG medium (35% increase, from 0.432 ± 0.034 to 0.582 ± 0.052, P < 0.05, Fig. 8A). In the presence of HG, cofilin phosphorylation was increased by about 20% (from 1.00 ± 0.046 to 1.21 ± 0.056, P < 0.05, n = 6). Metformin restored cofilin phosphorylation in HG

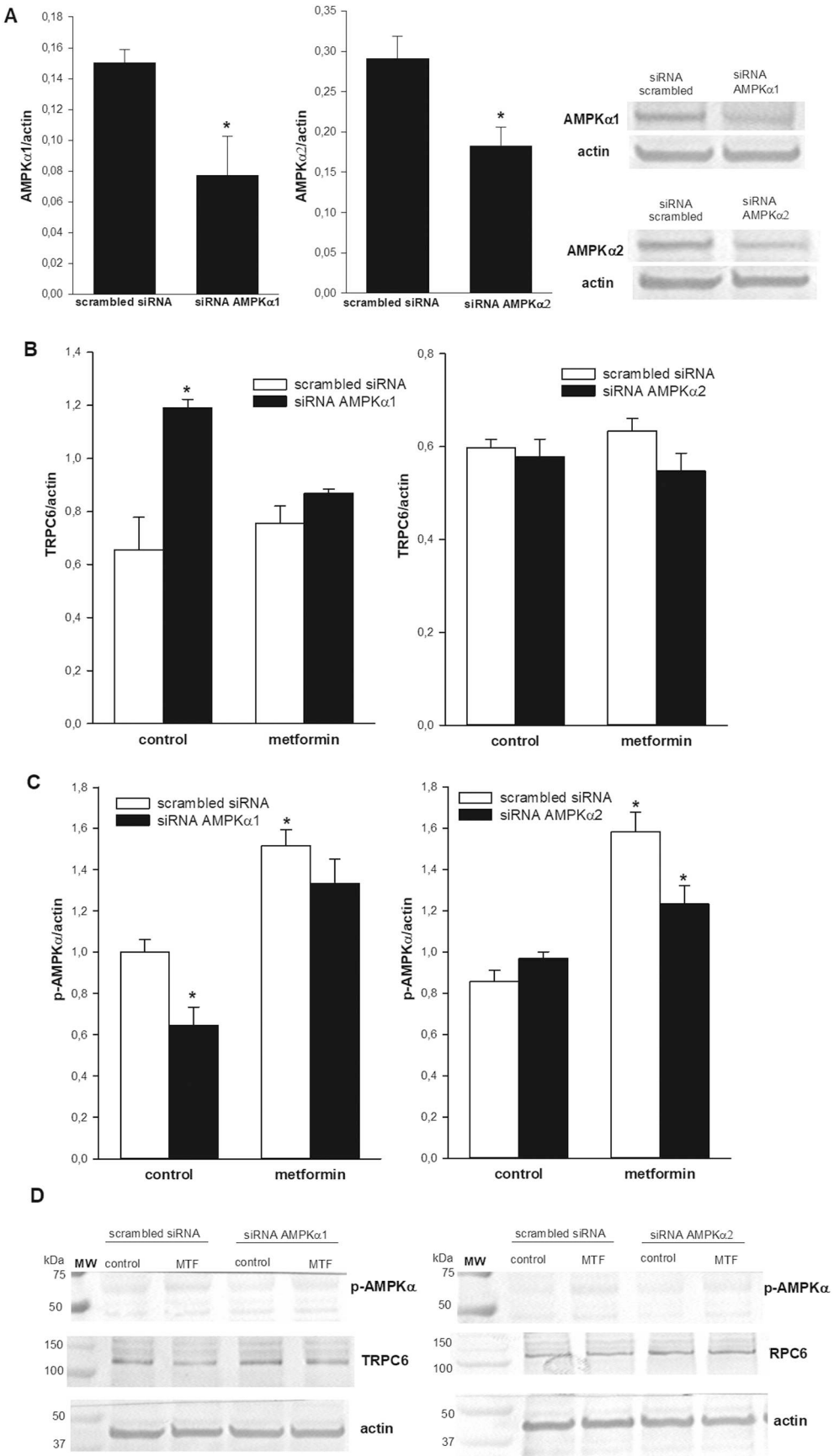

Fig. 3. The effects of AMPKα subunit downregulation and metformin treatment (MTF) on TRPC6 channel expression in podocytes. (A) AMPKα1 and AMPKα2 protein expression after transfection of target-specific small interfering RNA (siRNA) or scrambled siRNA. Densitometric measurements of AMPKα1 and AMPKα2 bands were normalized to the actin band. Values are the mean ± SEM of four independent experiments. *P < 0.05 versus transfection with scrambled siRNA. The effects of downregulating AMPKα1 or AMPKα2 in the presence of metformin (MTF, 2 mM, 5 days). We measured the levels of (B) TRPC6 protein content and (C) AMPK phosphorylation. Values are the mean ± SEM of four to five independent experiments. *P < 0.05 compared to control. (D) Representative immunoblots.

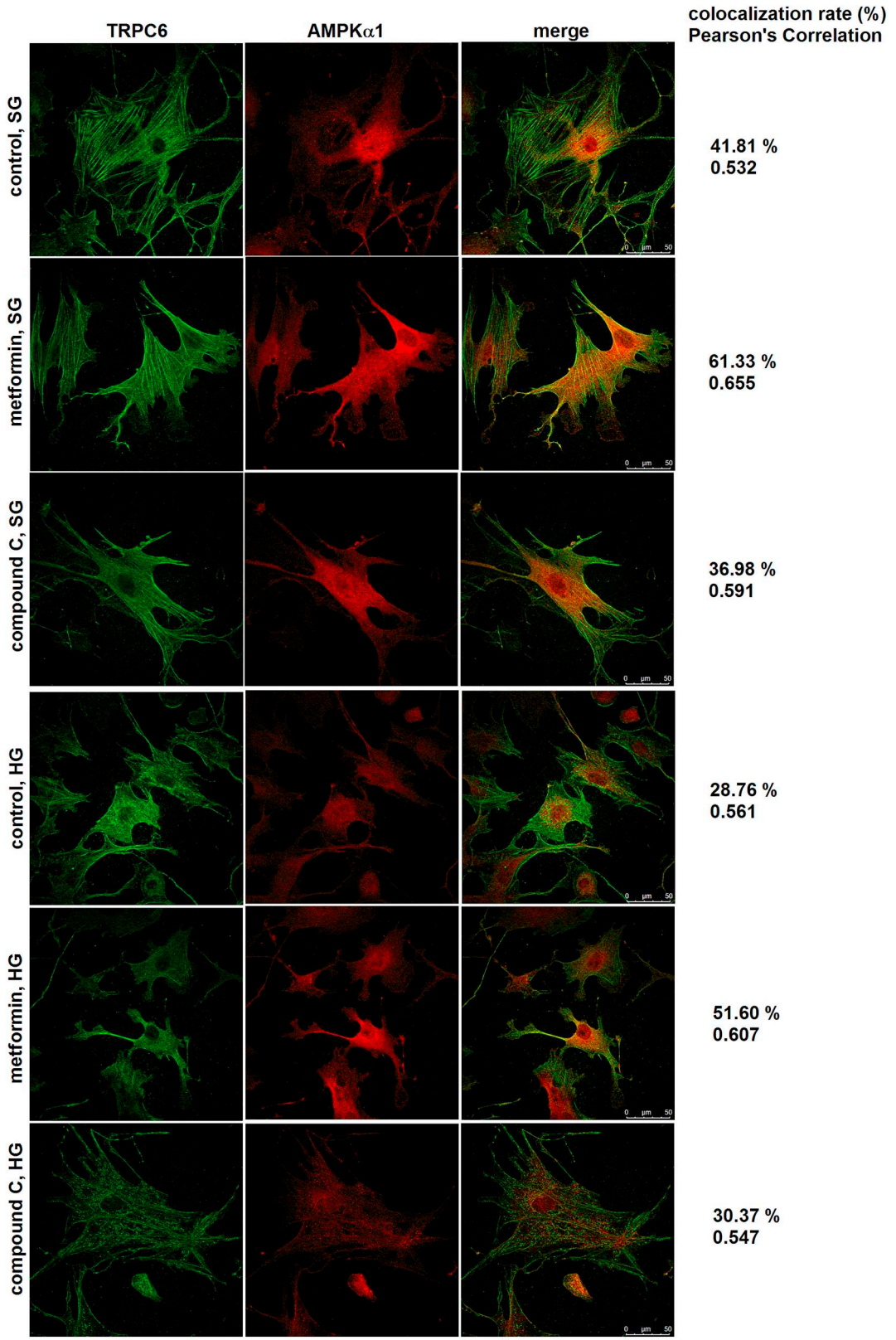

Fig. 4. Metformin-induced changes in the colocalization of AMPKα1 and TRPC6 channels in podocytes. Rat podocytes were seeded onto coverslips and incubated for five days with standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) concentrations in the presence of metformin or compound C. Cells were immunostained with anti-AMPKα1 and anti-TRPC6 antibodies, as indicated. Quantitative analyses of protein colocalizations were performed with LAS AF 3.3.0 software (n = 10–12). The pixel intensities were quantified and evaluated with Pearson's correlation to derive the colocalization rate (%).

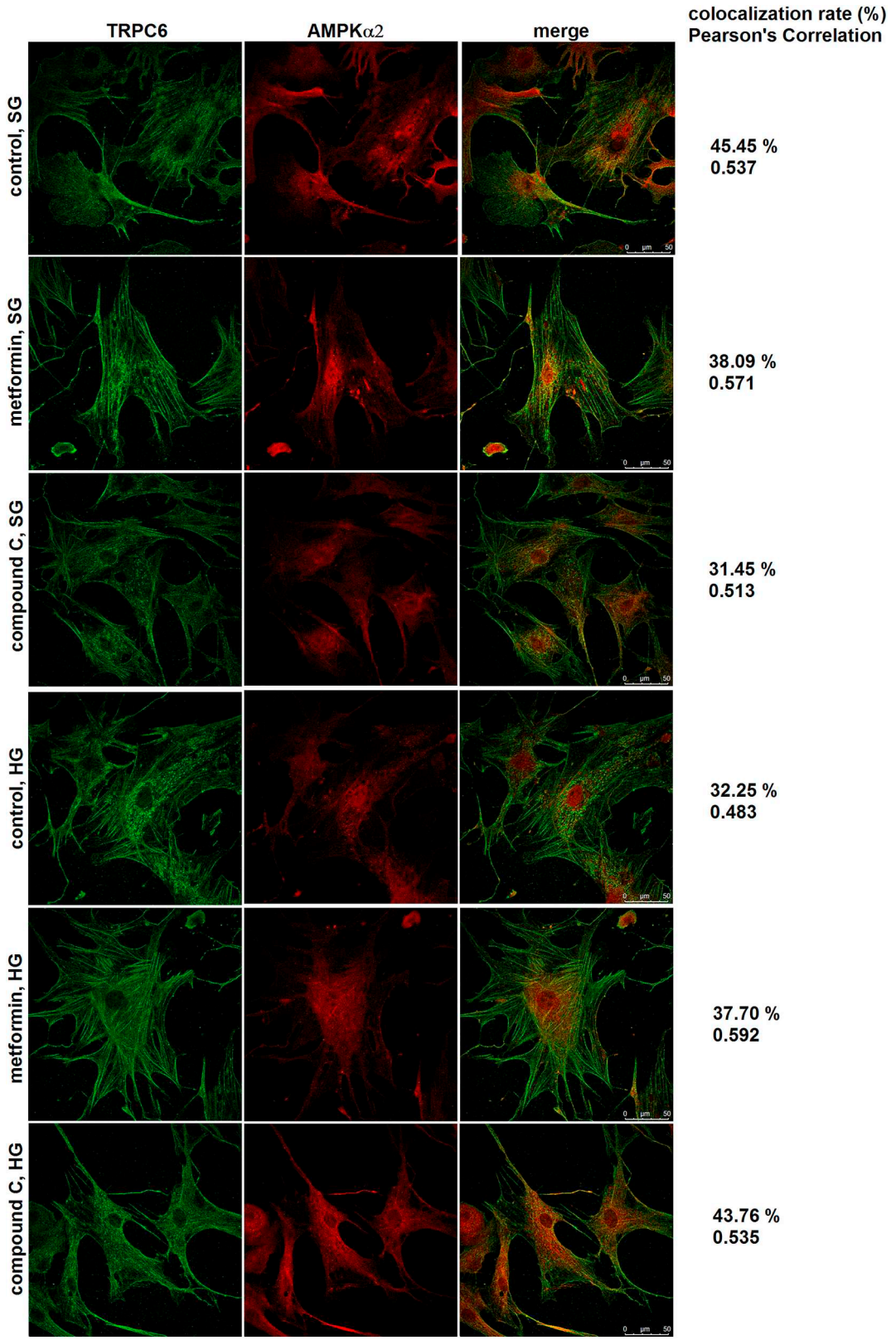

Fig. 5. Metformin did not affect the colocalization of AMPKα2 and TRPC6 channels in podocytes. Rat podocytes were seeded onto coverslips and incubated for five days with standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) concentrations in the presence of metformin or compound C. Cells were immunostained with anti-AMPKα2 and anti-TRPC6 antibodies, as indicated. Quantitative analyses of protein colocalizations were performed with LAS AF 3.3.0 software (n = 10–12). The pixel intensities were quantified and evaluated with Pearson's correlation to derive the colocalization rate (%).

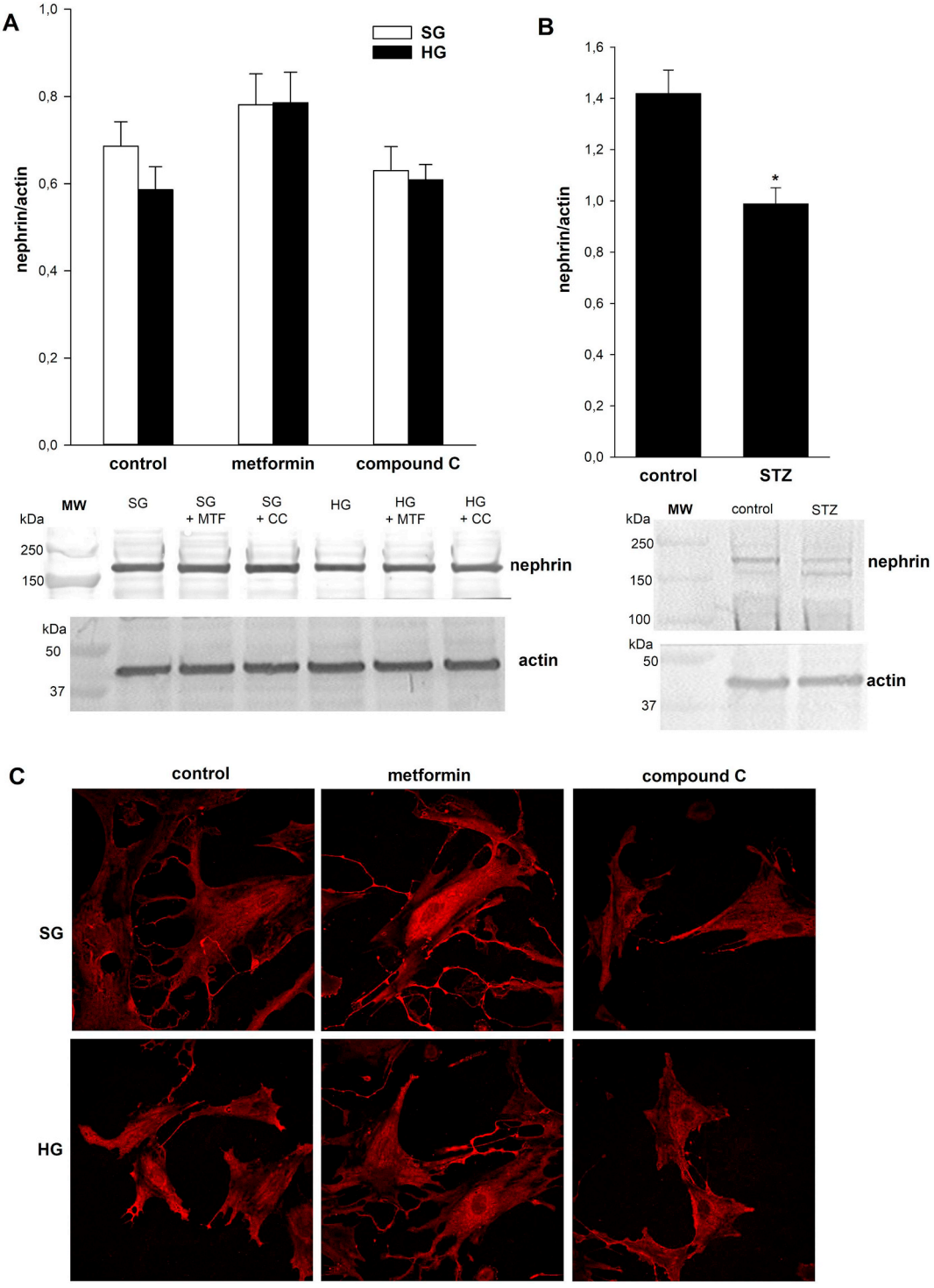

Fig. 6. The influence of AMPK activity on nephrin expression and localization in podocytes. (A) Metformin increased nephrin levels in podocytes cultured in standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) medium. (B) Diabetic conditions caused reductions in the amounts of nephrin protein in glomeruli isolated from control and streptozotocin (STZ)-treated rats. (C) The influence of metformin on the distribution of immunofluorescent nephrin in cultured rat podocytes. Values are the means ± SEMs (n = 4–6). *P < 0.05 vs. appropriate control. Densitometric quantifications were performed on the bands in the following lanes: (1) SG, (2) SG + metformin, (3) SG + compound C, (4) HG, (5) HG + metformin, (6) HG + compound C.

medium to values observed in SG (Fig. 8A). Compound C had no significant effect on PAK or cofilin phosphorylation in podocytes cultured in SG or HG. In addition, we observed that the effects of HG on PAK and cofilin phosphorylation in podocytes were recapitulated with hyperglycemia in glomeruli isolated from diabetic rats (Fig. 8B).

A quantitative analysis confirmed that HG concentrations and AMPK inhibition (compound C) increased F-actin immunostaining in the vicinity of the plasma membrane, but it had little effect on intracellular F-actin staining (Fig. 9). The effects of HG on the F-actin network were abolished by preincubating with the AMPK activator, metformin (Fig. 9).

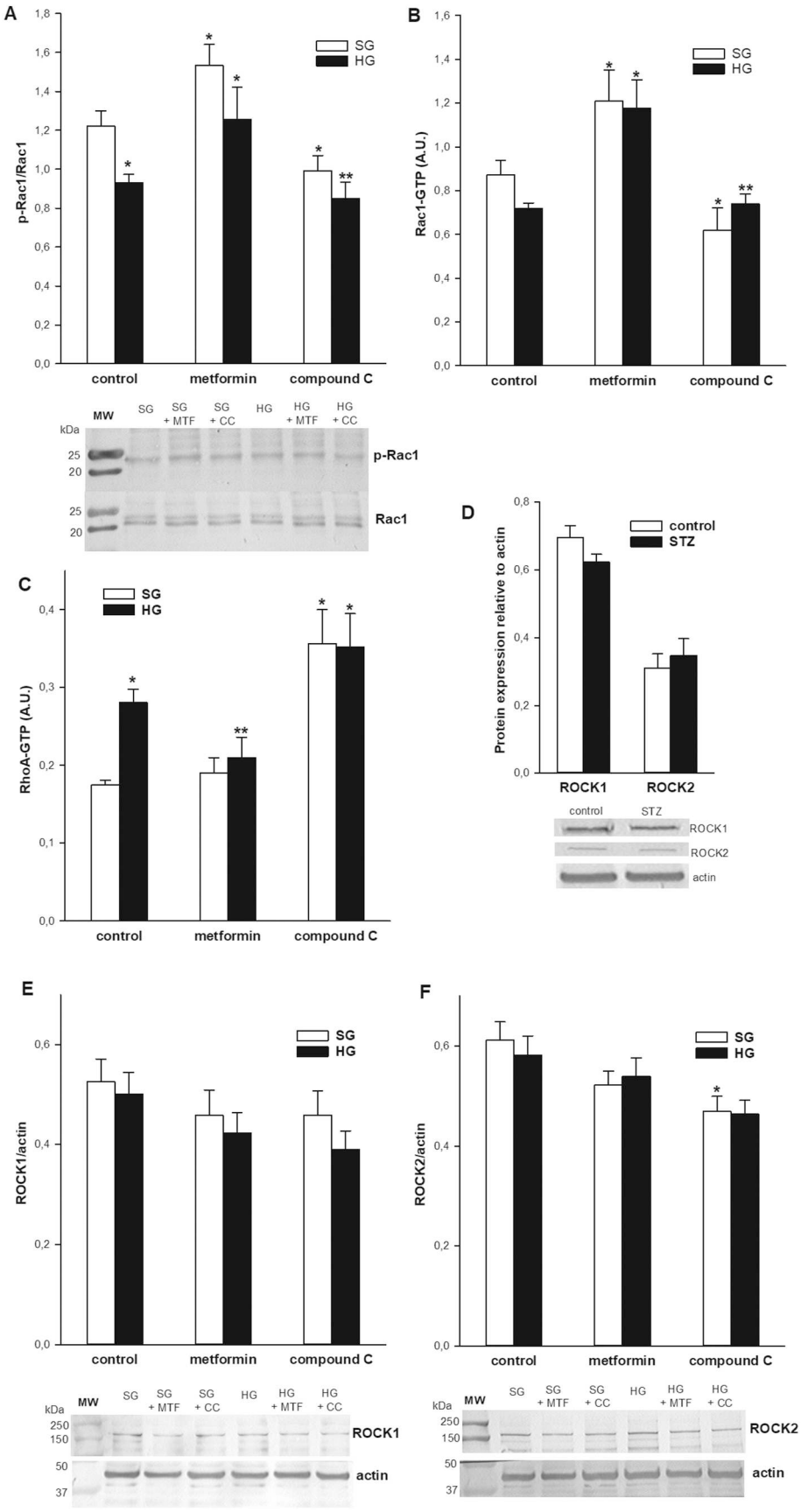

Fig. 7. The role of AMPK on HG-dependent regulation of Rho-family small GTPase activity and protein levels in podocytes. (A–C) The effects of AMPK activity on (A) Rac1serine-71 phosphorylation and on the regulation of (B) Rac1 and (C) RhoA activities. (D–F) Effects of diabetic conditions on the amounts of ROCK1 and ROCK2 proteins in (D) isolated glomeruli and (E,F) podocytes. Values are reported as the mean ± SEM of four to six independent experiments. *P < 0.05 vs. appropriate control, **P < 0.05 vs. SG. Densitometric quantifications were performed on the bands in the following lanes: (1) SG, (2) SG + metformin, (3) SG + compound C, (4) HG, (5) HG + metformin, (6) HG + compound C. Abbreviations: standard glucose (SG, 11 mM) or high glucose (HG, 30 mM).

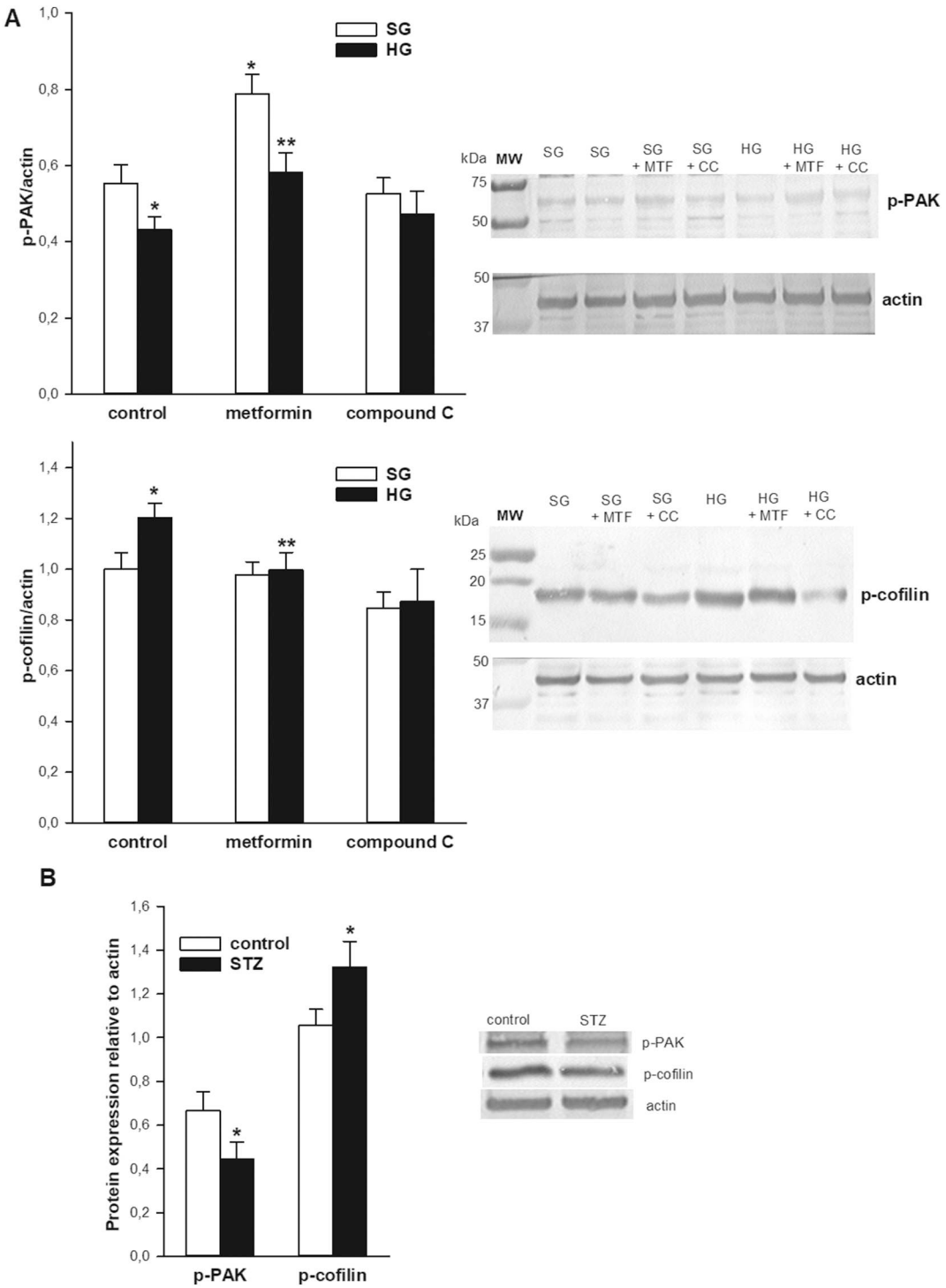

Fig. 8. The role of AMPK in HG-mediated phosphorylation of p21-activated kinase (PAK) and cofilin. Podocytes were cultured in the presence of standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) and treated with metformin or compound C. (A) Densitometric analysis of corresponding bands was performed, and values are reported as the ratios of band intensities for p-PAK (Thr^{423}) to PAK and for p-cofilin (Ser3) to cofilin. Diabetic conditions reduced PAK phosphorylation and increased cofilin phosphorylation in glomeruli isolated from control and diabetic (STZ) Wistar rats. Actin was used as a loading control. Values are the mean ± SEM of four to six independent experiments. *P < 0.05 compared to SG or control, **P < 0.05 compared to HG. Lanes: (1) SG, (2) SG + metformin, (3) SG + compound C, (4) HG, (5) HG + metformin, (6) HG + compound C.

3.6. Effects of AMPK activity on filtration barrier permeability

In diabetic conditions, significant increases were observed in glomerular capillary albumin permeability (P_{alb}: 0.562 ± 0.065 vs. control 0.098 ± 0.027, n = 14–16, P < 0.001, Fig. 10A) and in the permeability of a podocyte monolayer (132.39 ± 8.94 vs. control 85.61 ± 3.73 μg/ml, n = 6, P < 0.05, Fig. 10B). These effects were prevented by adding the AMPK activator, metformin (2 mM, 30 min pre-incubation), which resulted in a P_{alb} of 0.209 ± 0.108 (n = 14) and a FITC-albumin permeability of 65.32 ± 3.76 μg/ml (n = 6). The AMPK inhibitor, compound C (100 μM, 20 min), increased P_{alb} in control (0.677 ± 0.060 vs. control 0.098 ± 0.027, n = 14, P < 0.001) and diabetic glomeruli (0.803 ± 0.022 vs. control 0.562 ± 0.056, n = 14–16, P < 0.05), but did not influence podocyte

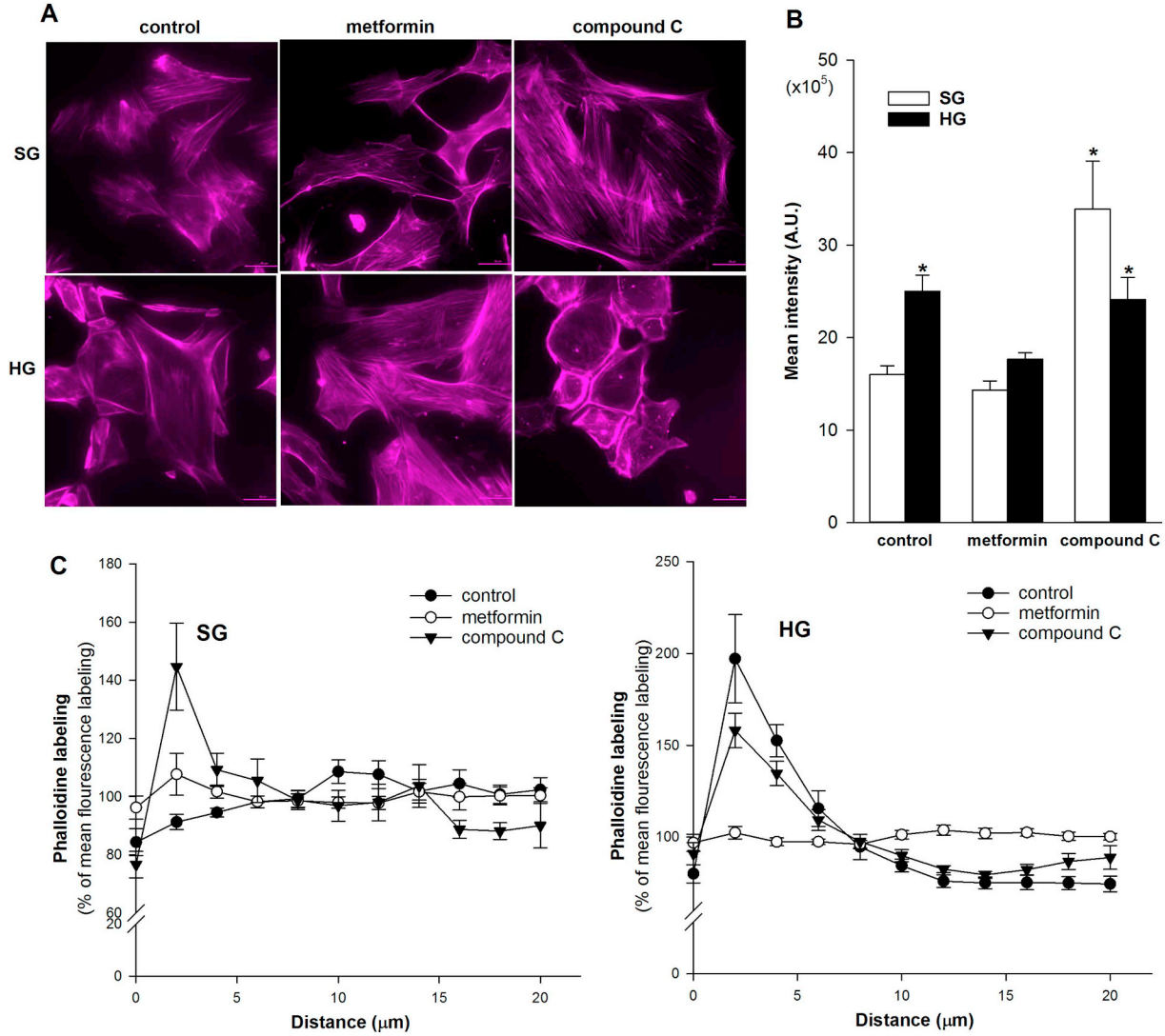

Fig. 9. AMPK activity regulates remodeling of the actin cytoskeleton in podocytes. (A) The F-actin network was labeled with isothiocyanate phalloidin and visualized with fluorescence microscopy. Cells were grown on coverslips, then incubated with standard glucose (SG, 11 mM) or high glucose (HG, 30 mM) in the presence or absence of an AMPK activator (metformin) or inhibitor (compound C). (B,C) The digitized fluorescence images of the F-actin network were used to generate the mean intensity and the fluorescence intensity profiles (from the basal membrane to the nucleus) with Cellsens image software. Values represent the mean ± SEM (n = 12–16).

permeability (Fig. 10). Next, we investigated the effects of AMPKα1 and AMPKα2 downregulation on podocyte permeability to albumin. As expected, downregulating AMPKα1 and AMPKα2 expression in podocytes induced significant increases in permeability (by approximately 64.3% and 57.8%, respectively) compared to controls treated with scrambled siRNA. Moreover, the effect of metformin on podocyte permeability was abolished in podocytes with AMPKα1 downregulation, but not in controls (Fig. 10C). Moreover we used the two TRPC6 inhibitors (SAR7334, 0.1 μM and SKF96365, 10 μM) to investigate the role of TRPC6 in the metformin-dependent decrease of podocytes permeability (Fig. 11). We observed that both inhibitors decrease of podocyte permeability to a level similar to metformin.

As shown in Fig. 12, a metformin infusion (50 mg/kg body weight) caused a reduction in the glomerular filtration rate (GFR) by 20% (0.84 ± 0.06 vs. 0.67 ± 0.05 ml/min, $P < 0.05$) in controls and by 25% (0.68 ± 0.06 vs. 0.51 ± 0.02 ml/min, $P < 0.05$) in diabetic rats. Furthermore, the metformin infusion increased cortical blood flow (CBF) by 18% (678 ± 46 vs. 575 ± 50 PU, $P < 0.001$) in diabetic rats, and it had no effect on control rats (618 ± 22 vs. 619 ± 22 PU). In this study we also monitored albumin level in urine sample collected in three 10-min periods. We observed increase of albumin level from 3.40 ± 0.63 to 9.05 ± 3.34 μg/ml ($n = 5$, $P < 0.05$) in diabetic rats. Additionally, the metformin infusion (30 min) into the abdominal aorta had no effect on albumin level neither in control nor in diabetic rats.

4. Discussion

This study showed that metformin reduced TRPC6 expression through AMPK activation and modulated cytoskeleton dynamics in podocytes under diabetic conditions.

First, metformin treatment increased AMPK activation and reduced TRPC6 channel expression in podocytes exposed to HG. Moreover, metformin regulated TRPC6 protein levels through AMPKα1 subunit activation. Second, metformin treatment restored nephrin expression and increased the amount of nephrin detected close to the cell surface in podocytes exposed to HG. Third, metformin remodeled the actin cytoskeleton, and consequently, regulated the permeability of the filtration barrier.

Metformin is the most widely used first-line drug for treating type 2 diabetes. It reduces hyperglycemia mostly by suppressing glucose production and release in the liver and by increasing insulin-stimulated glucose uptake in peripheral tissues, such as muscles. Recent studies have suggested that the therapeutic effects of metformin might be mediated by its action on AMPK [15,16]. In the current study, we

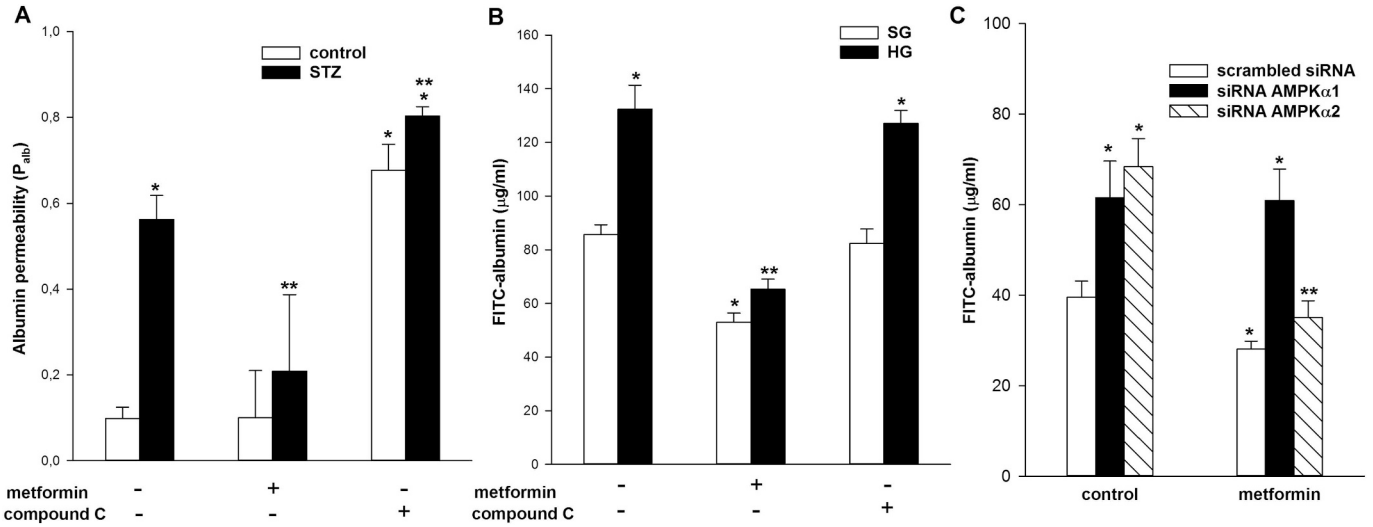

Fig. 10. Effects of AMPK activity on filtration barrier permeability. (A) The effects of AMPK activator (metformin, 2 mM, 20 min) and AMPK inhibitor (compound C, 100 μM, 20 min) on glomerular albumin permeability (P_{alb}) were measured. Control glomeruli were treated with equivalent volumes of buffer containing 5% bovine serum albumin. The values shown represent the mean ± SEM (n = 12–16 glomeruli from four rats). *P < 0.05 compared to control, **P < 0.05 compared to STZ. (B) The effects of metformin and compound C on albumin permeability across a podocyte monolayer. Results from four experiments are shown as the mean ± SEM. *P < 0.05 compared to SG, **P < 0.05 compared to HG. (C) The effects of down-regulating AMPKα1 and AMPKα2 on podocyte albumin permeability in the presence of metformin. The values shown represent the mean ± SEM of four independent experiments. *P < 0.05 compared to the appropriate control. Abbreviations: SG: standard glucose, 11 mM; HG: high glucose, 30 mM; STZ: streptozotocin.

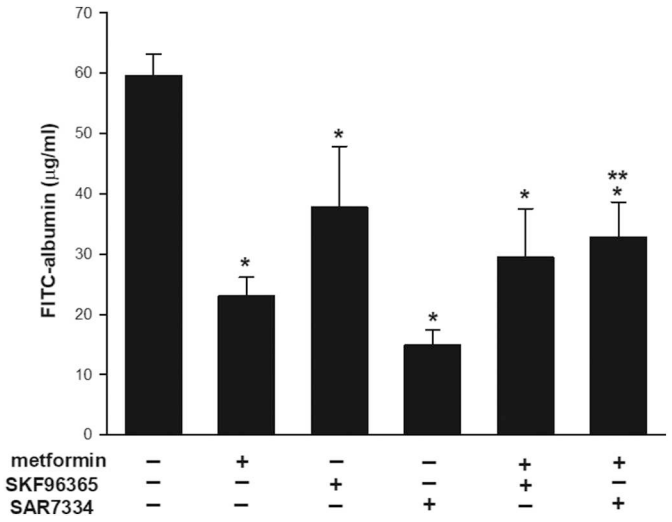

Fig. 11. The effects of the TRPC6 inhibitors on metformin-dependent decrease of podocyte permeability to albumin. Transepithelial permeability to albumin was evaluated by measuring the diffusion of FITC-labeled BSA across the podocyte monolayers. Cultured rat podocytes were stimulated with or without metformin (2 mM, 24 h) and TRPC6 channel inhibitors (SKF96365, 10 μM and SAR7334, 0.1 μM). Results from five experiments are shown as mean ± SEM. *P < 0.05 vs. control; **P < 0.05 vs. control with SAR7334.

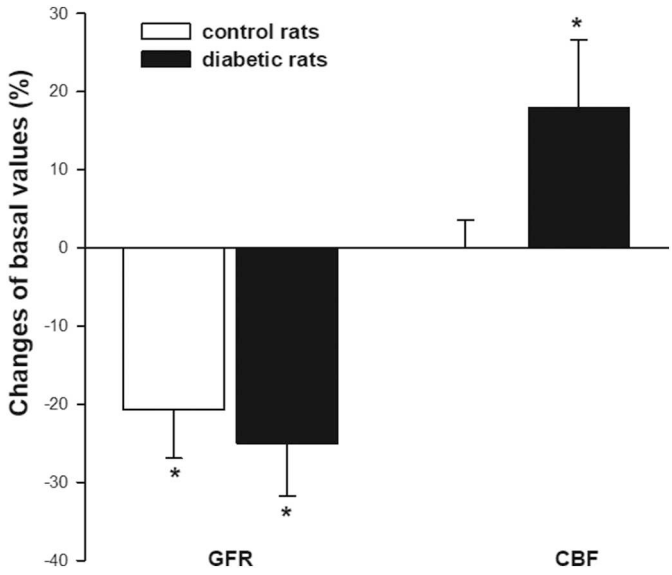

Fig. 12. Changes in glomerular filtration rate (GFR) and cortical blood flow (CBF) induced by metformin in control and diabetic rats. Data are the me an relative change ± SE. GFR values were derived from pooled averages of three 10-min baseline periods (control period) and three 10-min periods after metformin administration (50 mg/kg body weight). CBF values are averaged from three 5-min periods after metformin administration. *P < 0.05 vs. control period; n = 5–6 in each group.

observed that metformin treatment increased AMPK activation and reduced TRPC6 channel expression in podocytes exposed to HG. Moreover, we used metformin, as an AMPK activator, in in vivo studies involving STZ-treated rats, which are models of type 1 diabetes without primary insulin resistance. Our results showed that metformin, usually associated with treatment of type 2 diabetes, ameliorates also filtration barrier permeability in STZ rat models. Moreover, we observed that infusion of metformin into the abdominal aorta decreased the GFR but did not influence on albumin level in urine sample of control and diabetic rats. We suppose this is due to the short duration of the metformin infusion in vivo. Probably, this short-time period influences on kidney function (measured as a GFR) bud does not effect on marker of kidney damage (measured as an albumin level). Another authors demonstrated that long time administration with metformin (8–13 weeks) ameliorated diabetic nephropathy in rat models of diabetes [30,31].

Steady-state plasma level of metformin in humans is reported to be about 10–40 μM [32]. In the current study we used high concentration of metformin (2 mM) because its membrane permeability is slow and time-dependent process [33]. Therefore, such metformin concentration is required to see its effect on AMPK-dependent signaling in in vitro experiments.

However, a deeper understanding of how metformin alters AMPKα1

vs. AMPKα2 activation remains to be investigated. We demonstrated that the downregulation of AMPKα1 induced an increase in TRPC6 expression. However, the downregulation of AMPKα2 had no effect on TRPC6 expression. We also observed that metformin increased the co-localization of TRPC6 and the AMPKα1 subunit, but did not influence the amount of TRPC6 that colocalized with the AMPKα2 subunit. Therefore, we postulated that metformin reduced TRPC6 expression by activating the AMPKα1 subunit in podocytes exposed to HG. Another research group indicated that, in muscle cells, the inhibitory effects of metformin on fatty acid metabolism occurred via preferential phosphorylation of AMPKα1 [34]. Moreover, it was shown that metformin significantly increased AMPK phosphorylation and the activities of both AMPKα1 and AMPKα2 in skeletal muscle cells [34] and podocytes [16], where AMPK activation is associated with increased glucose uptake rates. Recently, we also showed a key role for the TRPC6 channel in mediating insulin-dependent activation of AMPKα2 and glucose uptake in cultured rat podocytes [35]. These studies suggested that the cellular effects of AMPK activation might depend on whether the AMPKα1 or AMPKα2 isoform was stimulated.

Other groups have demonstrated a link between TRPC channels and AMPK activation. For example, a TRPC1 knockdown in endothelial cells prevented PAR1 agonist peptide-induced AMPKα phosphorylation [36]. Moreover, both AMPK and TRPC3 took part in the same signaling pathway that affected the cytoskeletal network and erythrocyte survival [37]. We assumed that AMPK function depended on TRPC6-regulated calcium ion flux. A previous study showed that high glucose conditions could enhance calcium influx through TRPC6 activation, which then induced calcium-mediated podocyte dysfunction [38]. Therefore, we speculated that metformin activation of AMPKα1 could protect cells by inducing the inhibition of TRPC6 channel expression. Although measuring the total amount of TRPC6 detected in western blots may not relate straight to the actual activity of the channels in podocytes, or for that matter, on the amount of channel at the cell surface. Moreover we observed that TRPC6 inhibition by SAR7334 or SKF76365 did not influence on metformin protective effect on podocyte permeability.

Unfortunately, there are no specific inhibitors available for AMPK. Compound C (dorsomorphin) inhibits also several other kinases [39]. It was demonstrated that 40 μM of Compound C is required for total inhibition of AMPK activity in vitro. Moreover in this concentration Compound C inhibited several other kinases with a lower Km that AMPK. Therefore to inhibit AMPK activity, in our study we used also downregulation of AMPK by transfection with siRNA AMPKα1 and siRNA AMPKα2.

The slit diaphragm is a specialized cell-cell junction that is anchored to the actin cytoskeleton via a series of transmembrane proteins, including nephrin. The TRPC6 channel is located in the podocyte membrane and forms part of the signaling complex that interacts with nephrin, podocin, and other key players within the slit diaphragm [40,41]. Nephrin downregulation has been observed, by detecting protein and mRNA levels, in many human glomerular diseases, such as membranous nephropathy and diabetic kidney disease [42,43]. Moreover, increased urinary nephrin levels could reflect the degree of podocyte damage [44]. In the present work, we also found that the diabetic condition reduced nephrin expression in cultured rat podocytes. We showed that metformin treatment restored nephrin expression and increased the amount of nephrin detected close to the cell surface in podocytes exposed to HG. We also observed that the AMPK inhibitor, compound C, reduced nephrin protein levels to values observed with HG exposure. Taking these findings together, we concluded that AMPK activation improved nephrin expression in podocytes exposed to hyperglycemic conditions probably through decrease of total TRPC6 expression. Other groups have demonstrated that an 8-week metformin treatment reduced urinary albumin and nephrin excretion, increased renal tissue nephrin expression, and alleviated the fusion of podocyte processes in diabetic rats [30]. Moreover, another study reported that activating AMPK, sirtuin 1, and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) signaling pathways in mitochondria could relieve renal oxidative stress and improve nephrin expression in the podocytes of a diabetic rat model [45].

Furthermore, we previously demonstrated that metformin prevented glucose-induced oxidative stress in podocytes by inhibiting NADPH oxidase. We also showed a possible relationship between NADPH oxidase and AMPK activities [17]. On the other hand, it is known that the NOX2 and NOX4 isoforms of NADPH oxidase can induce the activation of TRPC6 in podocytes [38,46–48]. These findings suggested that the protective role of metformin on TRPC6 channel expression in hyperglycemia could have been due to AMPK-dependent suppression of NADPH oxidase activity (Fig. 13).

In the present study, we also demonstrated that the activation of AMPK by metformin induced a reorganization of F-actin via effects on the activities of Rho-family GTPases and their downstream targets. The Rho family has at least 20 distinct members, but of these, RhoA, Rac1, and Cdc42 have been the most extensively studied [49]. Rac1 is a major regulator of actin remodeling, and a cytoskeleton rearrangement is required for glucose transport in response to insulin [50]. Thus, our data suggested that Rac1, and its downstream signaling to the actin cytoskeleton, constituted an important dysfunctional pathway in insulin resistance, which we observed in hyperglycemia [51]. Moreover, we showed that metformin restored this signaling pathway in podocytes exposed to HG. Consistent with that finding, we recently demonstrated that the activation of AMPKα2 and TRPC6 were required for insulin-dependent stimulation of the Rac1 signaling pathway in podocytes [35].

Abnormal podocyte cytoskeletal rearrangement underlies not only the development of insulin resistance, but also the increased albumin permeability. Our study showed that metformin protects podocytes cytoskeleton which improves the function of podocyte in hyperglycemia. We demonstrated that AMPK activation induces a decrease of glomerular albumin permeability. The same effect we observed after inhibition or downregulation of TRPC6 channel [48]. It is noteworthy that the determination of glomerular volume may not directly reflect a measurement of glomerular albumin permeability, especially when the treatments affect TRPC6. This limitation is important because TRPC6 channel is expressed in many cells within glomeruli. For example, the contractile state of mesangial cells can influence glomerular volume. Moreover, it was reported that TRPC6 protein expression in mesangial cells was downregulated in hyperglycemic condition [52].

Overall, we demonstrated that metformin remodeled cytoskeleton

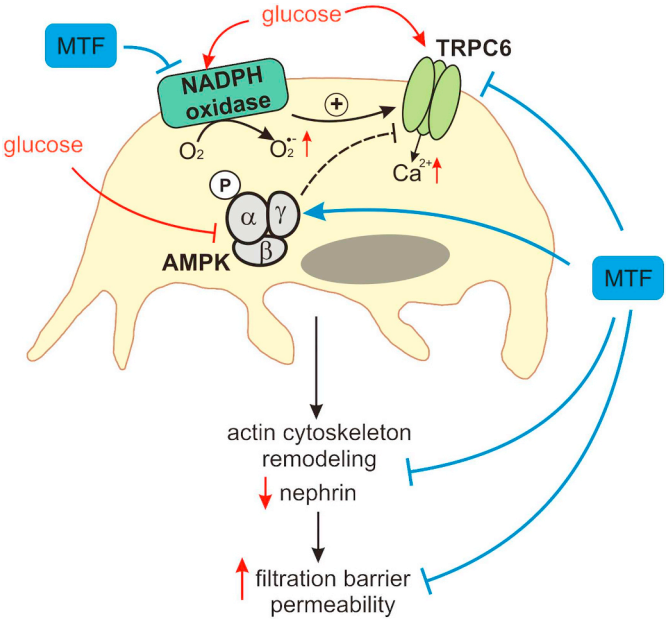

Fig. 13. Proposed mechanism of metformin action via the TRPC6-AMPK pathway in cultured rat podocytes. Abbreviations: MTF - metformin.

dynamics. In turn, this activity protected the podocyte slit diaphragm, and consequently, regulated the permeability of the filtration barrier in hyperglycemia.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

All authors declare no competing interests.

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Extracellular ATP modulates podocyte function through P2Y purinergic receptors and pleiotropic effects on AMPK and cAMP/PKA signaling pathways

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ABSTRACT

Podocytes and their foot processes interlinked by slit diaphragms, constitute a continuous outermost layer of the glomerular capillary and seem to be crucial for maintaining the integrity of the glomerular filtration barrier. Purinergic signaling is involved in a wide range of physiological processes in the renal system, including regulating glomerular filtration. We evaluated the role of nucleotide receptors in cultured rat podocytes using non-selective P2 receptor agonists and agonists specific for the $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors. The results showed that extracellular ATP evokes cAMP-dependent pathways through P2 receptors and influences remodeling of the podocyte cytoskeleton and podocyte permeability to albumin via coupling with RhoA signaling. Our findings highlight the relevance of the $P2Y_4$ receptor in protein kinase A–mediated signal transduction to the actin cytoskeleton. We observed increased cAMP concentration and decreased RhoA activity after treatment with a $P2Y_4$ agonist. Moreover, protein kinase A inhibitors reversed $P2Y_4$-induced changes in RhoA activity and intracellular F-actin staining. $P2Y_4$ stimulation resulted in enhanced AMPK phosphorylation and reduced reactive oxygen species generation. Our findings identify P2Y–PKA–RhoA signaling as the regulatory mechanism of the podocyte contractile apparatus and glomerular filtration. We describe a protection mechanism for the glomerular barrier linked to reduced oxidative stress and reestablished energy balance.

1. Introduction

The unusual permeability properties of the glomerular capillary wall depend on its three-layer structure, consisting of endothelial cells, the basement membrane, and podocytes. These components form the glomerular filtration barrier, a dynamic system undergoing constant remodeling [1]. Podocytes are terminally differentiated and highly specialized cells that cover the external surface of the glomerular capillary. The foot processes of podocytes along with the slit diaphragm form a dynamic structure that determines the final size-selectivity of the glomerular filtration barrier and thus constitutes an ultimate barrier to protein leakage. Podocytes, like smooth muscle cells, possess a functional contractile apparatus in foot processes that consists of F-actin, myosin, and α-actinin. Interactions among these proteins regulate proper podocyte shape and establish the size and properties of the glomerular filtration surface area. Actin cytoskeleton dysregulation, morphologically identified as foot process effacement, is closely associated with proteinuria [2,3].

AMP-activated protein kinase (AMPK) is considered an essential cellular energy sensor that is activated in response to intracellular ATP depletion. It plays a role in restoring energy homeostasis by activating ATP generation and inhibiting ATP-consuming pathways [4]. In a

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKK-β, calcium/calmodulin-dependent protein kinase beta; DCF, 2′,7′-dichlorodichydrofluorescein; LKB1, tumor-suppressor liver kinase B1; MLC, myosin light chain; PKA, protein kinase A; PKG, protein kinase G; ROS, reactive oxygen species; VASP, vasodilator-stimulated phosphoprotein.

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previous study, we showed that ATP simultaneously regulates AMPK activity and the NAD(P)H-dependent rate of $O^{2\bullet-}$ generation via P2 receptors in podocytes. Moreover, our results suggested that P2 receptor activation leads to calcium-dependent (via calcium/calmodulin-dependent protein kinase [CaMKK]-β) and calcium-independent (via tumor-suppressor liver kinase B1 [LKB1]) increases in AMPK activity [5].

Extracellular ATP is considered an essential autocrine and paracrine modulator of renal cell function [6]. Accumulating evidence suggests that activation of P2 receptors in the renal vasculature can produce vasoconstriction as well as vasodilation [7], implicating these receptors as essential in regulating glomerular filtration. Nucleotides are constitutively released into extracellular space from isolated glomeruli and podocytes, and their release increases in response to a wide range of stimuli, including mechanical stress, inflammation, and cell membrane damage [8]. Although nucleotide signaling in the kidney has been implicated in many pathological states, include diabetes and hypertension [9], the role in podocytes is still not clarified.

P2 receptors fall into two subgroups, P2X and P2Y, and are widely expressed in renal cells, including glomerular, renal tubular, renal vascular, and interstitial cells [6]. P2X receptors occur in seven subtypes ($P2X_{1–7}$) and are membrane cation channels gated by extracellular ATP. P2Y receptors are activated with differential selectivity by extracellular nucleotides, predominantly ATP and UTP, and occur as eight P2Y subtypes ($P2Y_{1, 2, 4, 6, 11–14}$) in mammals. P2Y receptors belong to the superfamily of G-coupled receptors whose primary function is transmission of extracellular stimuli into the cell through interaction with small G proteins, resulting in phospholipase C stimulation leading increased intracellular calcium, either activation or inhibition of adenylate cyclase converting ATP to cAMP [10]. Extracellular nucleotides also activate the endothelial isoform of nitric oxide (NO) synthase for NO generation, with subsequent activation of cytosolic guanylate cyclase and increased concentrations of cGMP in glomeruli and podocytes [5,9,10].

cAMP and cGMP, which regulate vasoactivity [11,12], exert many of their physiological effects by activating cAMP-dependent protein kinase A (PKA) and cGMP-dependent protein kinase G (PKG), respectively. These kinases in turn phosphorylate and regulate downstream protein targets including ion channels, enzymes, and transcription factors. We showed recently that the PKGIα isoform is expressed in cultured rat podocytes and that its activation results in actin reorganization and increased albumin permeability across the podocyte filtration layer [15]. Moreover, findings suggest that the cAMP–PKA signaling pathway in podocytes may regulate actin cytoskeleton organization and exert a glomerular protective action [16], although the precise molecular mechanism remains unclear.

Dependence between PKA and proteins related to the actin cytoskeleton has been reported in many cell types. cAMP-dependent kinase phosphorylates RhoA at Ser^{188}, inhibiting RhoA membrane translocation from the cytosol and resulting in inactivation [15,16]. RhoA is member of the Rho family of GTPases, which regulate many processes connected with cell motility and actin dynamics. Several lines of evidence have highlighted the role of RhoA/Rho-kinase signaling in pathogenesis of glomerular disease [19]. Of interest, Wang et al. [20] showed that either overactivation or inhibition of RhoA promotes podocyte injury, loss of foot processes, and albuminuria.

AMPK and PKA may communicate biochemically and act in concert as a signaling network to control cellular metabolism in vascular smooth muscle cells [21] and adipocytes [22]. Elevation of intracellular cAMP levels effectively increases AMPK phosphorylation through activation of PKA [23].

Recently, interest has grown in the role of superior protein kinases governing important signaling pathways in physiological barrier permeability. On this subject, we reported the above mentioned findings for PKG and AMPK regarding modifications to glomerular barrier permeability [24,25]. A previous study revealed that AMPK activation leads to remodeled cytoskeleton dynamics and reduced filtration barrier permeability. Moreover, this effect could be mediated by Rho family GTPases [26].

Based on this accumulating evidence, we investigated the role of P2 receptor activation in cAMP and cGMP and intracellular signaling, including the AMPK, PKA, and PKG pathways, in regulating actin cytoskeleton organization and filtration barrier permeability to albumin. Our findings offer new insights into the effects of P2Y-mediated extracellular ATP on podocyte physiology.

2. Methods

2.1. Preparation and culture of rat podocytes

All experimental procedures were performed in accordance with directive 2010/63/EU and were approved by the Local Bioethics Commission in Bydgoszcz. We used primary rat podocytes from Wistar female rats weighing 100–120 g, as described previously. In brief, animals were anesthetized with ketamine (65 mg/kg body weight i.p.) and xylazine (5 mg/kg body weight i.p.). Excised and crumbled kidneys were pressed through a system of sieves (160, 106, and 53 μm). Glomeruli were suspended in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin, plated in 75 cm2 type I collagen–coated culture flasks, and maintained at 37 °C in 95% air and 5% CO2 for 5–7 days. Outgrowing podocytes were sieved through Nylon mesh with 33-mm pores to remove the remaining glomerular cores. The resulting podocyte suspension was seeded in 24-well plates or culture flasks and cultivated at 37 °C in an atmosphere of 95% air and 5% CO2. Experiments were performed using podocytes that had been cultivated for 12–20 days. Podocyte phenotype and cell viability were determined as described previously [27] based on immunodetection methods using podocyte-specific antibodies against nephrin (ProSci Inc., Poway, CA, USA), podocin (Sigma-Aldrich), and podocalyxin (Sigma-Aldrich). Podocyte characteristics were maintained by all examined cells during incubation. On the day of the experiment podocytes were treated with ATP (100 μM) or the P2 ligands presented in Table 1 at a concentration of 10 μM. Cells were preincubated with PKA inhibitors (H89 or Rp-8-Br-cAMP, 10 μM) for 1 h.

2.2. Western blot analysis

Podocytes were treated with lysis buffer (1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol) in the presence of a protease and phosphatase inhibitor cocktail (Sigma-Aldrich) and homogenized at 4 °C by scraping. Equal amounts of protein extract (20 μg per well) were resolved on a 10% SDS-PAGE gel, transferred onto a PVDF membrane, and blocked with 3% fat-free milk in TBS (20 mM Tris-HCl, 140 mM NaCl). Then, the membrane was incubated overnight with primary antibodies against AMPKα (1:800, Cell Signaling Technology), P-AMPKα (1:800, Cell Signaling Technology), actin (1:10000, Sigma Aldrich), P-MLC (1:228, Cell Signaling Technology), RhoA (1:800, Santa Cruz Biotechnology), P-RhoA (1:200, Santa Cruz Biotechnology), vasodilator-stimulated phosphoprotein (VASP) (1:10000, Sigma Aldrich), P-VASP Ser157 (1:320, Santa Cruz Biotechnology), and P-VASP Ser239 (1:800, Abcam). For detection of primary antibodies, the membranes were incubated with the appropriate alkaline phosphatase–labeled

Table 1
P2 receptor agonists used for experiments and their specificity.

Ligand	Receptor specificity
ATP-γ-S	Non-selective P2 agonist
2-Methylthio-ATP	Non-selective P2 agonist
MRS 2365	Highly potent and selective $P2Y_1$ agonist
MRS 2768	Selective $P2Y_2$ agonist
MRS 4062	Selective $P2Y_4$ agonist

secondary antibodies. The protein bands were visualized using the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium system. Band density was measured quantitatively using the Quantity One program (Bio-Rad).

2.3. siRNA transfection

Podocytes were transfected with small-interfering RNAs (siRNAs) targeting AMPKα1 and AMPKα2 (Santa Cruz Biotechnology). Controls were transfected with non-silencing siRNA (scrambled siRNA, negative control). Cells were cultured in RPMI 1640 supplemented with 10% FBS. One day before transfection, the culture medium was removed and the cells cultivated in antibiotic-free RPMI 1640 supplemented with 10% FBS. The cells were transfected with siRNAs and the siRNA Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's protocol. In brief, the targeted siRNA or scrambled siRNA was diluted in Transfection Medium to a final concentration of 80 nM, then mixed with siRNA Transfection Reagent and incubated for 30 min at room temperature. The transfection mixture was added to the Transfection Medium, mixed gently, and added to the podocytes for 7 h. Next, growth medium containing 2-fold higher FBS and antibiotics was added for another 24 h. After transfection, gene silencing was assessed at the protein level by western blotting. The efficiency of transfection was 53% for AMPKα1 (0.08 vs. 0.15) and 59% for AMPKα2 (0.17 vs. 0.29).

2.4. Immunofluorescence

Podocytes were seeded on type I collagen–coated coverslips (Becton Dickinson Labware, Beckton, UK) and cultured in RPMI 1640 supplemented with 10% FBS. Next, cells were preserved in 4% paraformaldehyde in PBS for 20 min at room temperature and permeabilized in 0.3% Triton X-100 for 2 min. Coverslips were placed in blocking buffer (PBS containing 2% FBS, 2% BSA, and 0.2% fish gelatin) for 1 h. Cells were incubated with the primary antibodies anti-PKA (1:15, Santa Cruz Biotechnology), anti-PKG (1:15, Santa Cruz Biotechnology), and anti-RhoA (1:40, Origene Technologies) for 2 h, washed three times with cold PBS, and incubated with the secondary antibodies Alexa Fluor 488 (1:750) or Alexa Fluor 546 (1:750) for 60 min. Actin was stained with Alexa Fluor 594 phalloidin (1:200). Specimens were imaged using a confocal laser scanning microscope and NIS-Elements software (Nikon) with a 63× oil immersion lens.

2.5. Measurement of intracellular ROS

Reactive oxygen species (ROS) generation was measured with using fluoroprobe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCDA). The cell rapidly takes up this compound and uses intracellular esterases to convert it to 2′,7′-dichlorodichydrofluorescein (DCF), which can be visualized by fluorescence at 525 nm. Podocytes after incubation with P2 agonists were treated H_2DCFDA (10 μM) for 30 min at 37 °C, in air-5% CO_2, in the dark. The fluorescence emission of DCF was measured using a spectrofluorometer (LS55, Perkin Elmer) with excitation/emission wavelengths set at 485/525 nm. The results of intracellular ROS generation are represented in arbitrary units (A.U.).

2.6. RhoA activity and cGMP and cAMP concentrations

RhoA activation was measured in the supernatant using a commercially available RhoA G-LISA Activation Assay Kit (BK121; Cytoskeleton, Inc.). A commercially available kit (Cyclic GMP Competitive ELISA Kit, Thermo Fischer Scientific) was used to determine the concentration of cGMP in lysed podocytes. To determine cAMP concentrations in lysed podocytes, we also used a commercially available kit (Cyclic AMP Competitive ELISA Kit, Thermo Fischer Scientific).

2.7. Permeability assay

To estimate transepithelial permeability to albumin, we measured the diffusion of FITC-labeled BSA (Sigma) across a podocyte monolayer, as described previously [16]. Briefly, rat podocytes were seeded onto type IV collagen–coated cell-cultured inserts with a 3-μm membrane pore size and were placed in 24-well plates. The cells were allowed to differentiate for one week and used for experiments between 7 and 15 days post-seeding. Before use in experiments, the podocytes were washed twice with PBS and medium on both sides, and the insert was replaced with serum-free RPMI 1640 medium. After 2 h, the medium in the upper compartment was replaced with 0.2 ml of fresh serum-free medium, and the medium in the lower compartment was replaced with 1.2 ml serum-free medium containing 1 mg/ml FITC-albumin. After 1 h of incubation, 200 ml of the solution in the upper chamber was transferred to a 96-well plate, and the absorbance of the FITC-albumin was evaluated at 490 nm using a plate spectrophotometer (BioTekEL808).

2.8. Statistical analyses

All statistical analyses were performed in GraphPad Prism 8. The Shapiro–Wilk test was used to determine whether parametric or nonparametric tests should be implemented. Statistical significance was determined by repeated-measures ANOVA with a Tukey post hoc test or paired *t*-test. The results are presented as mean ± SEM with $p < .05$ considered to indicate significance.

3. Results

3.1. The role of P2 signaling in AMPK phosphorylation in cultured rat podocytes

Initially, we investigated the effect of extracellular ATP on AMPKα phosphorylation at three time points (after 1, 3, and 10 min of incubation). A maximal effect was reached in the first minute, at which point we observed doubling of AMPK phosphorylation level (from 0.40 ± 0.07 to 0.90 ± 0.12, $p < .05$; Fig. 1A). There was a tendency to a decrease after that point, with a return to the control level at 10 min. For this reason, we used 1 min for all subsequent experiments.

Previous research has shown that podocytes express $P2Y_{1,2,4,6,11}$ [28], so we chose P2 receptor agonists for this work (Table 1). Extracellular ATP is a highly non-selective ligand for nucleotide receptors and is rapidly metabolized by ecto-nucleotidases that are localized on the cell surface. Accordingly, we also used poorly metabolized ATP analogues such as ATP-γ-S and 2-methylthio-ATP in these experiments.

We checked the degree of AMPK phosphorylation in the presence of P2 ligands. All compounds that we applied increased AMPK phosphorylation level, and we observed ~75% augmentation in the presence extracellular ATP (from 0.6 ± 0.05 to 1.05 ± 0.09, $p < .05$; Fig. 1B). We identified similar results with the non-selective agonists ATP-γ-S (70%, $p < .05$) and 2-methylthio-ATP (45%, $p < .05$), but detected the strongest effect with the selective $P2Y_4$ agonist MRS 4062 (~93%, $p < .05$). We demonstrated comparable dependence for acetyl-CoA carboxylase (ACC), the substrate for AMPK (Fig. 1C). These results suggest that purinergic signaling is involved in regulating AMPK phosphorylation.

3.2. The effect of P2 signaling on intracellular ROS production in podocytes

In a previous study, we showed that activation AMPK by metformin leads to an increased extracellular ATP concentration and subsequent activation of P2 receptors [29]. We hypothesized that this mechanism is coupled with decreasing ROS production through inhibition of NAD(P)H oxidase. To test this hypothesis, here we assessed DCF-sensitive ROS production in cultured podocytes and found antioxidant action on the

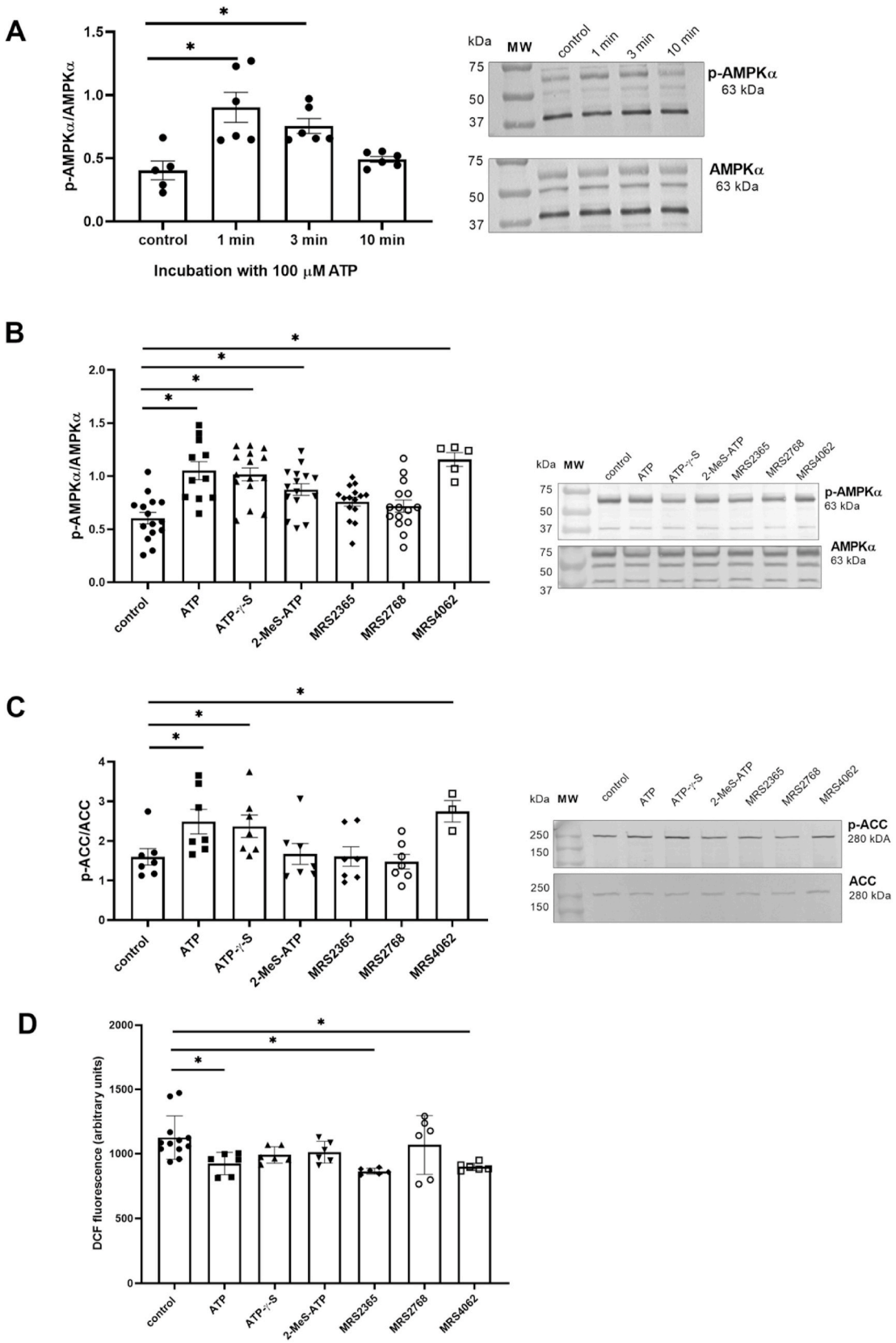


Fig. 1. (A) Time course of the effects of ATP on AMPK phosphorylation in podocytes. (B) Effects of P2 receptor agonists on phosphorylation of AMPK and (C) ACC in podocytes. The effect of P2 signaling on intracellular production of ROS in cultured rat podocytes. (D) Cells were incubated with 100 μM ATP for 1, 3, or 10 min (A) or for 1 min with ATP (100 μM), ATP-γ-S, 2-MeS-ATP, MRS2365, MRS2768, or MRS4062 at a concentration of 10 μM (B–D). Densitometric quantification of podocyte lysates was performed to determine the degree of AMPK (A, B) and ACC (C) phosphorylation. ROS generation was measured by DCF (D). Values are the mean ± SEM (n = 3–15). *p < .05 compared to control.

cells with short-term incubation with P2 agonists. Podocytes were treated with ATP (100 μM) and P2 receptor agonist (10 μM) at 1 min. Significantly reduced ROS production was observed in the presence of extracellular ATP (~17%) and selective agonists of $P2Y_1$ (23%) and $P2Y_4$ (20%) compared to control ($p < .05$; Fig. 1D).

3.3. The influence of P2 signaling on cAMP-dependent and cGMP-dependent pathways in podocytes

P2Y receptors in response to nucleotide stimulation trigger production of secondary messengers, i.e., inositol phosphate, Ca^{2+}, and products of the activity of adenylyl and guanylyl cyclases [30]. To assess the potential impact of activation of the PKA and PKG pathways on nucleotide signaling, we determined cAMP and cGMP concentrations in cultured rat podocytes. The results showed increased cAMP concentration in podocytes treated with almost all non-selective agonists but a significant increase with ATP (from 3.12 ± 0.25 to 4.28 ± 0.36, $p < .05$; Fig. 2A) and a selective agonist of the $P2Y_4$ receptor (from 1.81 ± 0.19 to 3.09 ± 0.44, $p < .05$). Of interest, 2-MeS-ATP led to reduced cAMP concentrations ($p < .05$; Fig. 2A). As shown in Fig. 2B, ATP and non-selective analogues of ATP markedly enhanced cGMP concentrations compared to control, in contrast to selective agonists, which reduced cGMP concentrations ($p < .05$). However, the most inhibitory action was by a selective agonist for $P2Y_4$, which suppressed cGMP concentration by half (from 1.22 ± 0.17 to 0.6 ± 0.08). Based on these results, we inferred that P2 receptor activation turns on cAMP- and cGMP-dependent pathways in podocytes, whereas P2Y stimulation extinguishes PKG-dependent signaling and intensifies PKA-dependent

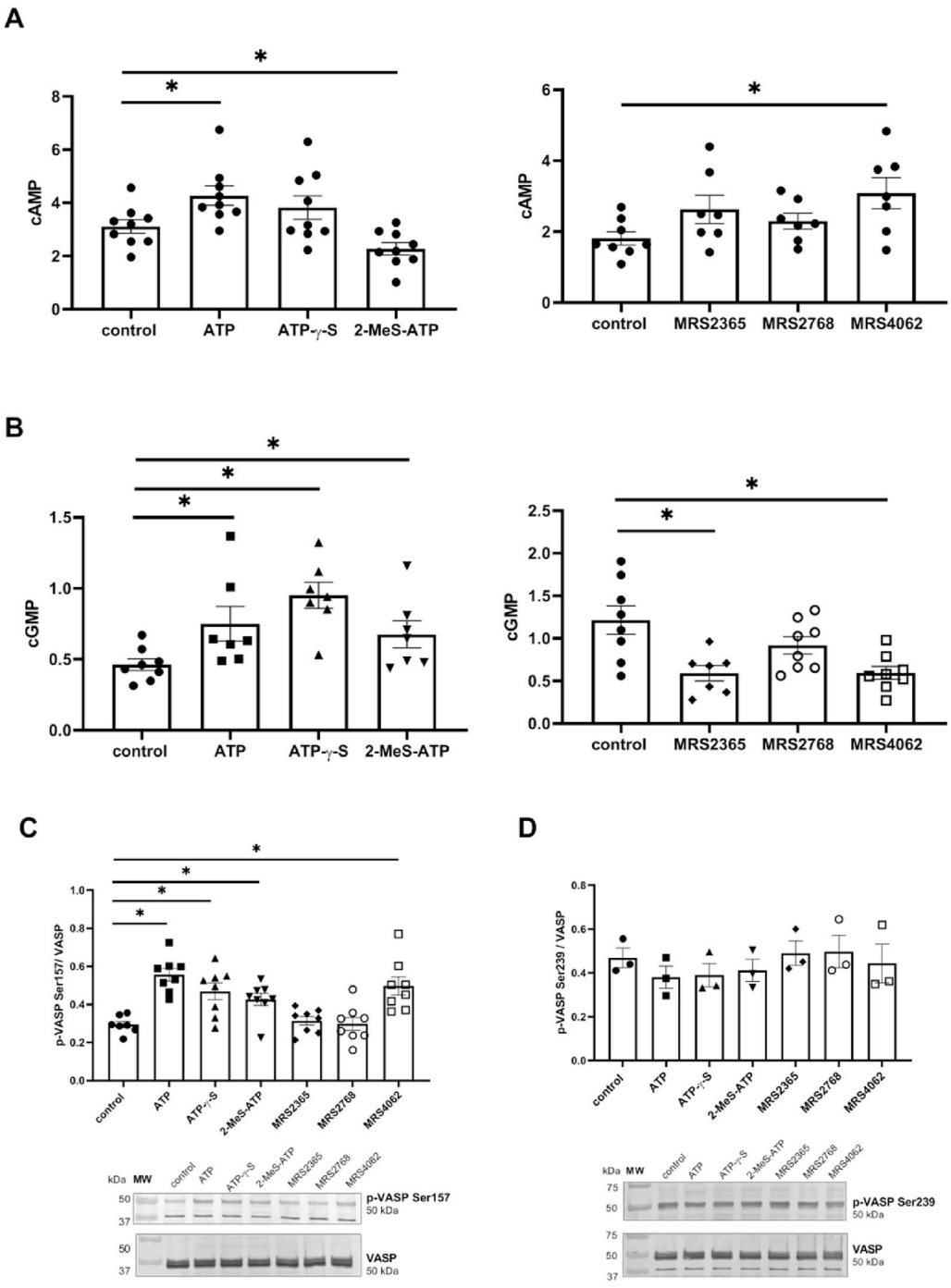


Fig. 2. (A) The influence of P2 signaling on cAMP and (B) cGMP concentrations in podocytes. Cells were exposed to P2 agonists, and cell lysates were analyzed using commercial tests. Results are reported in pmol/mg protein as the mean ± SEM (n = 7–9). *$p < .05$ compared to control. The effect of purinergic signaling on VASP phosphorylation at Ser157 (C) and Ser239 (D) in podocytes is shown. Cells were incubated with P2 agonists for 1 min. Densitometric quantifications were performed on the bands in the following lanes: control, ATP, ATP-γ-S, 2-MeS-ATP, MRS2365, MRS2768, and MRS4062. Values are presented as the mean ± SEM (n = 3–8). *$p < .05$ compared to control.

signaling.

VASP, an actin regulatory protein involved in a range of processes related to cytoskeleton remodeling, is a known substrate of cAMP- and cGMP-dependent protein kinases, which mainly phosphorylate the protein at Ser157 and Ser239, respectively [31]. For this reason, we checked the phosphorylation level of VASP in podocytes after a short-term incubation with ATP analogues. We found changes in VASP phosphorylation level at Ser157. The addition of 100 μM ATP induced a 1.9-fold increase over the untreated control (from 0.3 ± 0.02 to 0.56 ± 0.03, $p < .05$; Fig. 2C). VASP phosphorylation at Ser157 was likewise increased in the presence of the poorly metabolized ATP analogues, the non-selective ATP-γ-S (1.6-fold) and 2MeSATP (1.5-fold) and selective $P2Y_4$ receptor agonist (1.7-fold). We observed no changes with MRS2365 and MRS2768. Furthermore, none of the agonists altered VASP phosphorylation levels at Ser239 (Fig. 2D). Thus, stimulation of nucleotide receptors seems to trigger pathways associated with PKA in podocytes, and the $P2Y_4$ receptor seems to play a central role in this process. Moreover, this pathway appears to be coupled to VASP.

3.4. The influence of P2 signaling on the RhoA pathway

Recently, new findings about the Rho family of GTPases have led to increased interest in these proteins because of their crucial role in remodeling the actin cytoskeleton, which drives many dynamic processes in cell morphology and metabolism. We focused on RhoA, which regulates the assembly of contractile actin and myosin filaments and may be related to adjustments in the size of the glomerular filtration surface, adaptation to environmental changes, and maintaining an intact filtration barrier [32]. In the current study, we assessed RhoA activity in the presence of P2 agonists. We observed an ~18% decline in activity after ATP stimulation (0.41 ± 0.05 vs. control 0.56 ± 0.04, $p < .05$; Fig. 3A) in cultured podocytes. Exposing the podocytes to ATP-γ-S led to about a 35% reduction in activity (from 0.56 ± 0.04 to 0.35 ± 0.01) and a reduction of about 25% with the P2Y4 agonist (from 0.55 ± 0.01 to 0.4 ± 0.02).

The contractile effect of RhoA results from activation of Rho-dependent kinase, which phosphorylates and inactivates the myosin light chain (MLC) phosphatase. This action leads to inhibition of its function, thus allowing for increased levels of phosphorylated MLC and contraction at a constant Ca^{2+} [33]. Therefore, we examined phosphorylation of MLC in cultured podocytes under treatment with the different compounds, as shown in Fig. 3B. We observed decreased phosphorylation of MLC in the presence of all agonists, significantly so with extracellular ATP and the selective agonist for $P2Y_4$. These results for Rho activity and MLC phosphorylation are consistent and suggest that stimulation of nucleotide signaling in podocytes promotes relaxation of the actin cytoskeleton through suppression of the RhoA pathway.

3.5. The interplay between PKA and AMPK in podocytes

The results of these analyses led us to hypothesize that activation of purinergic receptors, notably $P2Y_4$, is involved in signal transduction to the actin cytoskeleton in podocytes, via RhoA and MLC proteins and dependent on PKA. Moreover, this pathway seems to be coupled with

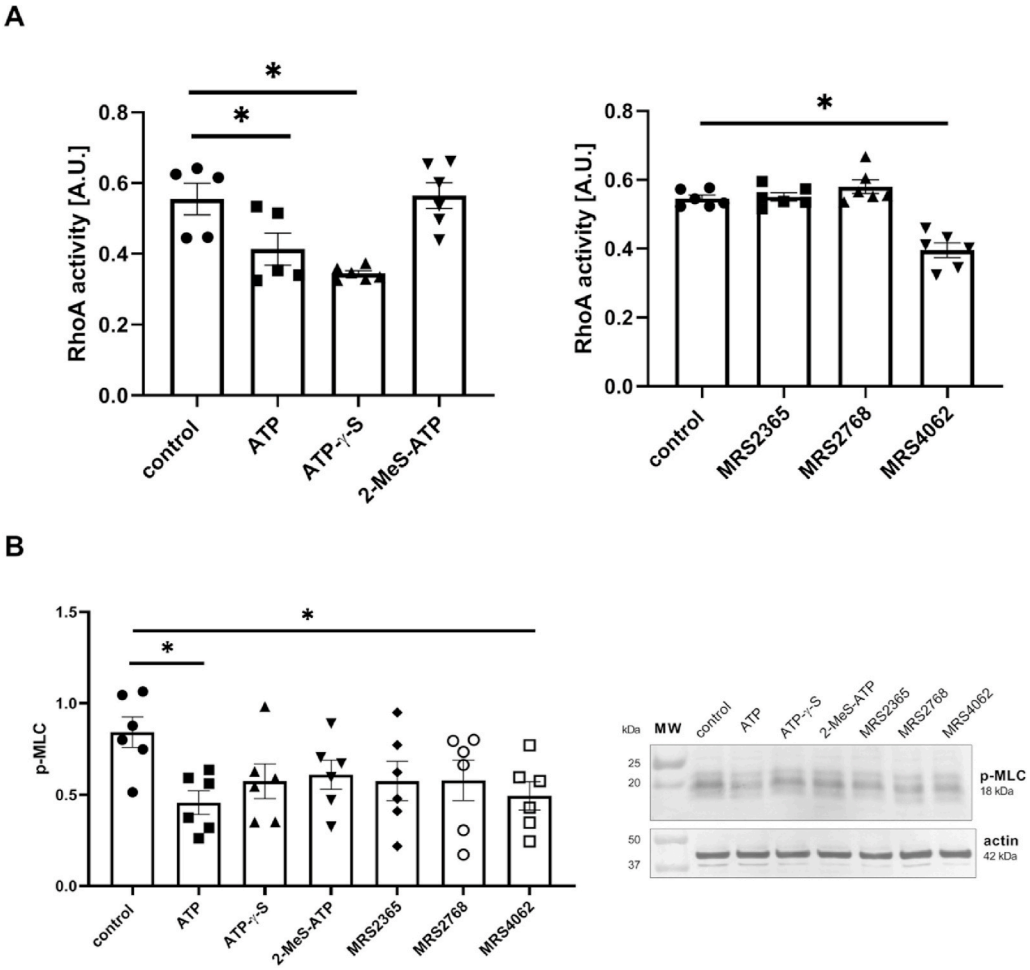


Fig. 3. (A) The influence of P2 signaling on RhoA activity and (B) MLC phosphorylation. Cells were exposed to P2 agonists for 1 min. Cell lysates were evaluated using a G-LISA assay (A) or immunoblotted with anti-p-MLC and anti-actin antibodies (B). Values are mean ± SEM (n = 5–6). *$p < .05$ compared to control.

AMPK. For these reasons, we decided to inhibit PKA and test its hypothesized involvement using two PKA inhibitors: H89 and Rp-8-Br-cAMP.

At the beginning, we assessed AMPK phosphorylation in the presence of ATP and the $P2Y_4$ receptor agonist with and without PKA inhibitors. Exposing the podocytes to both ATP and MRS4062 alone resulted in increased AMPK phosphorylation ($p < .05$; Fig. 4A and B). Similarly, ATP and MRS4062 enhanced AMPK phosphorylation levels in podocytes preincubated with PKA inhibitors vs controls. Densitometric quantification of the degree of VASP phosphorylation at Ser157 confirmed the involvement of PKA in P2Y-induced regulation of actin cytoskeleton organization. As shown in Fig. 4C and D, phosphorylation of VASP at Ser157 was increased in the presence of both ATP and the $P2Y_4$ agonist. Using H-89 resulted in a significant decline in phosphorylation level, which was restored to control levels in the presence of ATP ($p < .05$; Fig. 4C).

In addition, we assessed whether AMPK can affect PKA activity. For this purpose we examined the role of AMPK activity in regulating VASP phosphorylation at Ser157. We used two activators of AMPK (metformin at 2 mM and AICAR at 0.5 mM; 2 h incubation) or downregulated AMPK expression using siRNA. We observed no change in the degree of VASP phosphorylation under these conditions (Fig. 5A, C). Activation of AMPK also did not affect the location of VASP protein in podocytes (Fig. 5B). All of these results indicated a lack of mutual regulation between these proteins.

3.6. The role of PKA inhibition in the RhoA signaling pathway

Next, we studied whether blocking PKA signaling affects the P2Y-mediated inhibition of RhoA activity. Preincubation of podocytes with inhibitors of PKA prevented ATP- and MRS4062-dependent inhibition of RhoA activity ($p < .05$; Fig. 6A and B). These findings were reflected in western blot analysis of phosphorylation levels. We noted that pretreating podocytes with H-89 and Rp-8-Br-cAMP eliminated responses to ATP and MRS4062 ($p < .05$; Fig. 6C and D). Using inhibitors of PKA also reversed the degree of MLC phosphorylation suppressed by ATP and MRS4062 to control levels ($p < .05$; Fig. 6E and F). Moreover, immunofluorescence experiments showed that ATP and MRS4062 caused substantial changes in the subcellular localization of PKA in cultured rat podocytes (Fig. 7). With both agonists, the intensity of PKA immunostaining increased close to the cell surface. The quantitative analysis confirmed that both agonists decreased the colocalization of PKA and RhoA proteins. Pretreatment of podocytes with H-89 induced translocation of RhoA close the cell membrane and inhibited agonist-dependent translocation of PKA. These results are consistent with those of the RhoA activity studies. The activation was accompanied by the translocation of this protein to the cell membrane. However, quantitative analysis showed that neither agonist influenced the colocalization of PKGIα and RhoA proteins (Fig. 8). The present findings confirmed our hypothesis of a role for PKA in mediating P2Y-induced inhibition of the Rho pathway in remodeling of the actin cytoskeleton.

3.7. The effect of inhibition of PKA on remodeling of F-actin cytoskeleton

A quantitative analysis of intracellular F-actin staining showed that ATP and MRS4062 contributed to intensified signals. We observed that both agonists directly increased F-actin immunostaining close to the plasma membrane. These effects on the F-actin network were prevented by preincubating podocytes with PKA inhibitor (Fig. 9). We postulated that PKA plays an essential role in the regulation of the podocyte contractile apparatus.

3.8. Effects of P2 signaling on podocyte permeability to albumin

To check the impact of nucleotide signaling on glomerular filtration, we measured transmembrane flux for albumin across the podocyte monolayer in the presence of P2 agonists. We found that albumin permeation was about 2-fold higher after ATP-γ-S (187.8 ± 9.8) and 2-MeS-ATP (172.8 ± 18.4) treatment and approximately 1.5-fold higher in the presence of ATP (142.9 ± 9.8) and selective agonists for $P2Y_1$ (137.2 ± 4.5), $P2Y_2$ (149.3 ± 13), and $P2Y_4$ (130.6 ± 8.4) compared to controls (90.5 ± 2 μg/ml; $p < .05$; Fig. 10). Additionally, the effects of ATP and MRS4062 were abolished in podocytes preincubated with PKA inhibitors. These results suggested that P2Y agonists led to increased albumin permeability through the podocyte filtration barrier via PKA-dependent activation.

4. Discussion

In this study, we found that nucleotide receptor stimulation initiates the cascade of events that leads to reorganization of the actin cytoskeleton in podocytes and consequent changes in glomerular filtration barrier permeability. Moreover, we characterized molecular crosstalk between cAMP-dependent and RhoA signaling pathways. Our results suggest that a P2Y-induced mechanism of RhoA inactivation is coupled with PKA activation. In addition, we have shown the important role of $P2Y_4$ in the regulation of the cAMP–PKA–RhoA signaling pathway in podocytes (Fig. 11).

The present study implicates the cAMP-dependent pathway as a prominent link between purinergic signaling and actin regulation. We demonstrated that PKA activity is tightly associated with RhoA function and cytoskeleton dynamics. Inhibition of PKA resulted in reversion of P2Y-mediated suppression of RhoA activity. Furthermore, analysis of immunofluorescence staining and the degree of colocalization between PKA and RhoA confirmed interaction of these proteins. Based on our findings, PKA is required for P2Y-induced actin remodeling and consequently filtration barrier permeability regulation.

In preliminary research, we assumed that P2-mediated activation depends on PKA and PKG. Measurement of cAMP and cGMP concentration in podocytes verified the validity of this assumption. Our observations that non-selective P2 ligands stimulate PKGIα-dependent pathways in cultured podocytes are consistent with recent studies [11]. Nonetheless, experiments with specific ligands of P2Y receptors indicated PKA as the main effector of this signaling. Results related to cAMP concentration, VASP phosphorylation, and immunostaining expressly showed predominant relevance for PKA in P2Y-mediated transduction of cellular signals in podocytes.

Whereas our understanding of P2 signaling in kidneys is well known, information is limited concerning P2 signaling in podocytes. It is worth emphasizing that little is known about individual nucleotide receptor characteristics in podocytes. Previous research has been related to the whole subgroup of receptors, and analysis of particular receptors is needed, although specific synthesized ligands can be difficult to access. Non-selective agonists could activate a wide range of receptors, both P2X and P2Y, so current knowledge is incomplete. Findings from another group indicated that mobilization of intracellular calcium via phospholipase C is the main pathway mediated by extracellular ATP and purinergic signaling in podocytes. Furthermore, $P2Y_1$ [34], $P2Y_2$, and $P2Y_6$ receptors [35] are essential in this process. Our observations could suggest that Ca^{2+} is not the only second messenger that P2Y stimulates in podocytes. We found that P2Y stimulation increases cAMP concentration and that $P2Y_4$ seems to be most efficient in this process. Burford et al. [36] suggested that purinergic propagation of a Ca^{2+} wave constitutes a key pathogenic mechanism in podocyte injury. Given the protective effect of cAMP on endothelial function, $P2Y_4$-mediated activation of cAMP-dependent pathways might have compensatory properties.

The current literature provides increasing evidence for the role of purinergic signaling in renal hemodynamics [37]. The presence of P2 receptors in glomerular cells might be crucial in metabolic adaptation to changes in the hydrostatic pressure of filtered blood. Several groups have reported a relaxing effect on glomeruli of extracellular nucleotides,

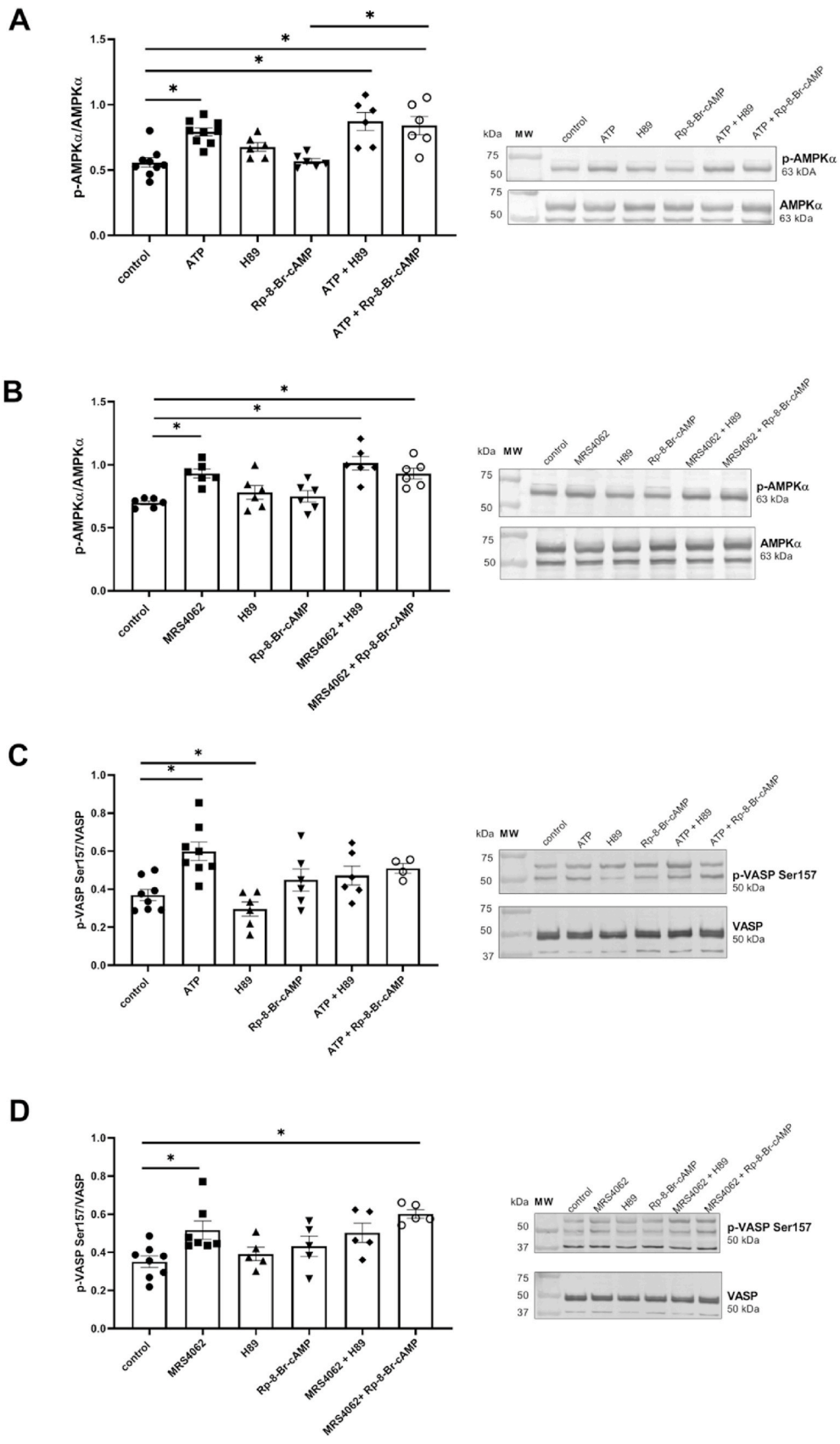


Fig. 4. The effect of inhibition of PKA on phosphorylation of AMPK (A, B) and VASP at Ser157 in the presence of ATP (A, C) and MRS4062 (B, D). Podocytes were pre-incubated with H89 or Rp-8-Br-cAMP (10 μM, 1 h) and incubated for 1 min with 100 μM ATP (A, C) or 10 μM MRS4062 (B, D). Cell lysates were analyzed by immunoblotting using anti-AMPKα and anti-p-AMPKα (Thr172) (A, B) or anti-VASP and anti-p-VASP (Ser157) antibodies (C, D). Values are reported as mean ± SEM (n = 4–9). *p < .05 compared to control.

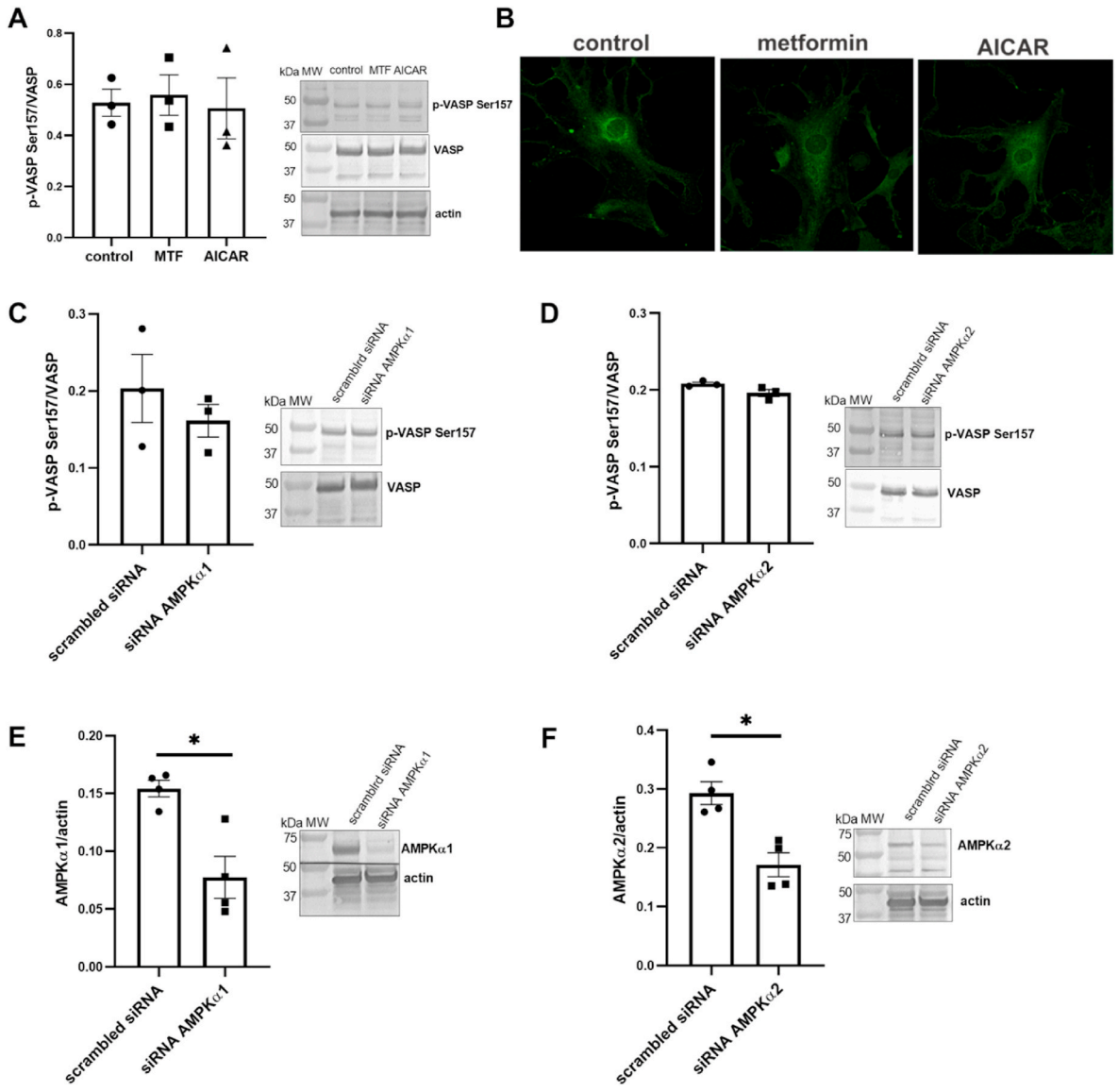


Fig. 5. The effect of pharmacological activation of AMPK on phosphorylation level (A) and cellular localization (B) of p-VASP Ser157. Podocytes were incubated with metformin (MTF, 2 mM) and AICAR (0.5 mM) for 2 h. (C, D) The effect of AMPKα subunit downregulation on phosphorylation of VASP at Ser157. (E, F) AMPKα1 and AMPKα2 protein expression after transfection of target-specific siRNA or scrambled siRNA. Densitometric measurement of AMPKα1 and AMPKα2 bands were normalized to the actin band. Statistical significance was determined by paired *t*-test. Values are mean ± SEM (n = 3–4). *p < .05 compared to transfection with scrambled siRNA.

via the P2–NO–cGMP molecular pathway [11]. In agreement with other reports, our results may suggest that P2 stimulation relaxes the glomerular filtration barrier. Supporting this idea is the suppression of RhoA activity and decreased phosphorylation of MLC, which promote the relaxant effect. Several lines of evidence have suggested a vasodilatation effect of inhibitory RhoA phosphorylation at Ser188 through AMPK [38] and PKG [39]. We recently demonstrated that activation of AMPK does not influence RhoA activity in podocytes cultured in standard glucose medium. We also previously found that an inhibitor of AMPK (compound C) or a high glucose concentration increases RhoA activity [26]. In the current work, we showed a close relationship between RhoA and PKA, modified by purinergic signaling. Recent reports suggest that RhoA activity in podocytes must be tightly controlled to maintain podocyte function. A growing body of evidence highlights activation of RhoA in podocytes as leading to loss of cellular processes, cell contraction, and proteinuria [40]. Thus, we could infer that P2Y-mediated suppression of RhoA activity might have protective properties. Additionally, this mechanism is directed by PKA, which attenuates albuminuria and foot process effacement and prevents podocyte apoptosis. Several other groups have confirmed a mechanism that we previously proposed of a negative feedback loop between PKA and RhoA, which is recognized as a protective pathway against inflammatory injury [41].

Given the linkage between extracellular nucleotides and microvascular permeability, we also assessed the influence of P2Y activation on the glomerular filter surface. Our results showed enhanced transmembrane flux for albumin across the podocyte monolayer after P2 agonist treatment, confirming a relaxant effect of P2 signaling on the glomerular filtration barrier. The fact that the presence of PKA inhibitors prevented this effect indicates involvement of PKA in controlling albumin permeability. This association strongly supports the hypothesis that cAMP is critical to maintaining normal permeability. Indeed, cyclase adenylate knockout mice are susceptible to developing proteinuria [42], and reduced cAMP levels lead to increased microvessel permeability [43]. Moreover, cAMP is an intracellular signaling molecule linked to improved barrier function in vascular endothelial cells [17]. Although

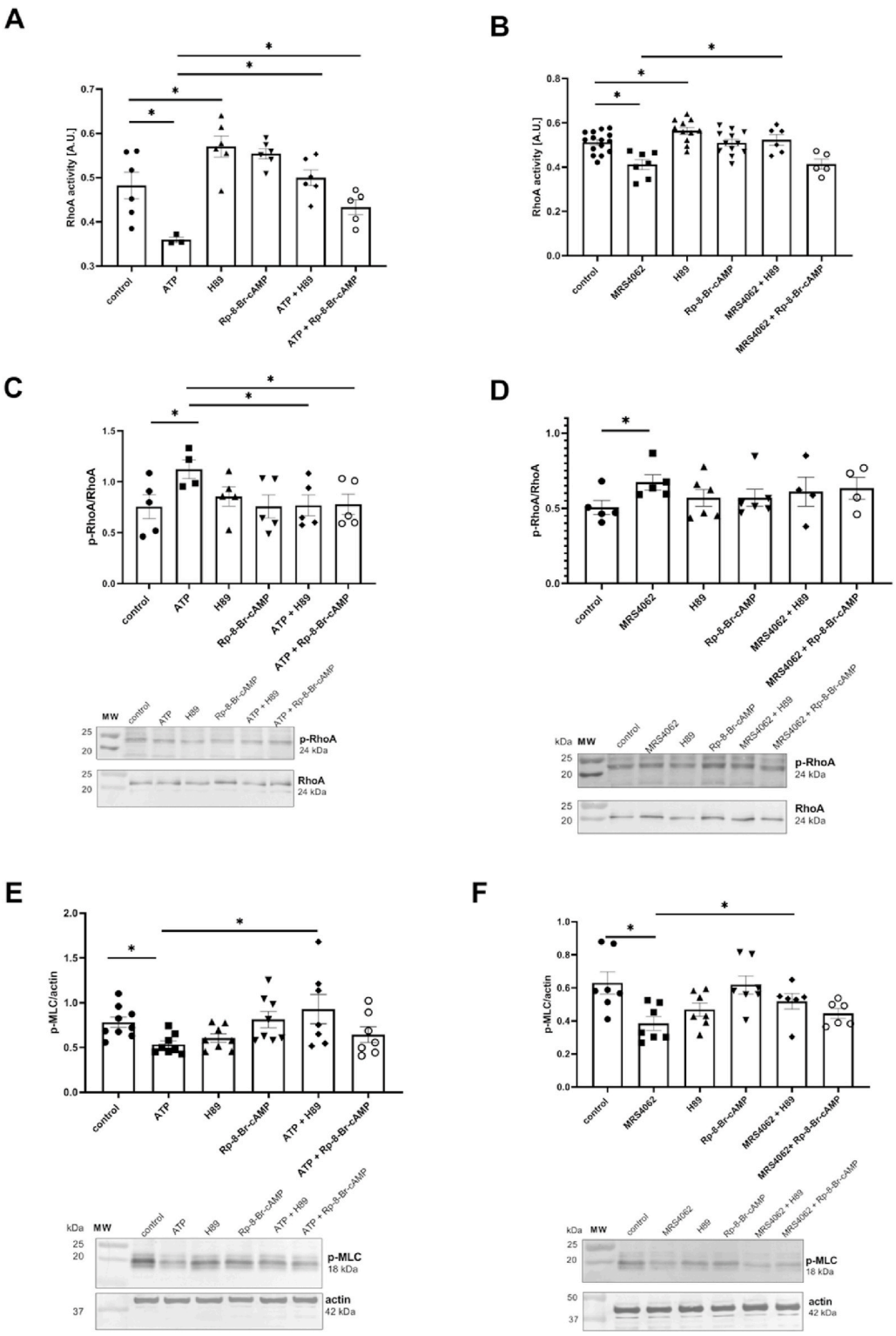


Fig. 6. The role of PKA inhibition in the RhoA signaling pathway. The effect of inhibition of PKA on RhoA activity in the presence of ATP (A) and MRS4062 (B). The influence of inhibition of PKA on RhoA phosphorylation in the presence of ATP (C) and MRS4062 (D). The influence of inhibition of PKA on MLC phosphorylation in the presence ATP (E) and MRS4062 (F). Podocytes were pre-incubated with H89 or Rp-8-Br-cAMP (10 μM, 1 h) and incubated for 1 min with 100 μM ATP (A, C, E) or 10 μM MRS4062 (B, D, F). Cell lysates were assessed using a G-LISA assay (A, B) or immunoblotted with anti-RhoA and anti-p-RhoA (C, D) or anti-MLC and anti-actin antibodies (E, F). Values are reported as the mean ± SEM (n = 3–12). *p < .05 compared to control.

cAMP reduces glomerular albumin permeability by direct action on the glomerulus [44], reports demonstrate a role for cAMP in inhibiting Ca^{2+}- sensitization through a Ca^{2+}-independent mechanism and promoting a relaxant effect. Our present findings support those of Hayashi et al. [45], who reported changes in force tension in human detrusor smooth muscle, mediated by suppression of the RhoA pathway and putative modulation of MLC kinase or phosphatase activity. Based on this accumulated evidence and the current findings, we infer that P2 stimulation during podocyte injury could preserve glomerular filtration through activation of PKA–RhoA pathways.

We suggest that P2 receptor activation increases AMPK phosphorylation in podocytes, and we propose a repair process of restoring energy homeostasis after nucleotide imbalance. This mechanism might play an important role in pathological conditions associated with cell damage and efflux of cytoplasmic nucleotides into the extracellular space. Our current findings in this regard are in agreement with our previously published results [5]. Here we showed that modulation of energy metabolism through AMPK activation is associated with $P2Y_1$, $P2Y_2$, and

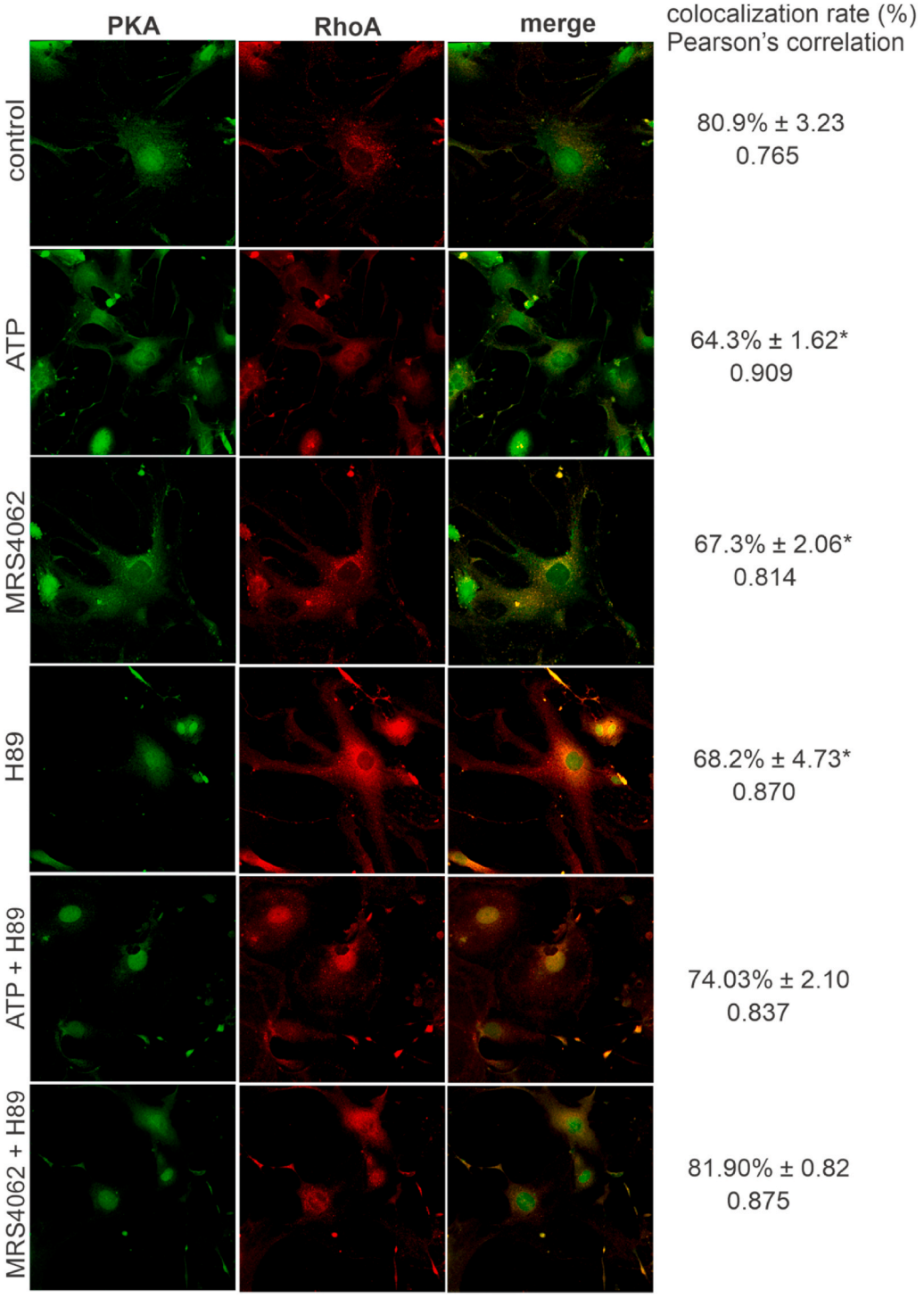


Fig. 7. P2Y-mediated changes in subcellular localization of PKA and RhoA in cultured rat podocytes. Cells seeded onto coverslips were pre-incubated with H89 (10 μM, 1 h) and incubated for 1 min with 100 μM ATP or 10 μM MRS4062. Cells were immunostained with anti-PKA and anti-RhoA antibodies. Quantitative analysis of protein colocalization was performed with NIS-Elements software (Nikon). The pixel intensities were quantified, and results are given as Pearson's correlation coefficients and colocalization rates (%). Values are reported as the mean ± SEM (n = 10–14). *p < .05 compared to control.

especially $P2Y_4$ receptors. Da Silva et al. [46] proposed a similar association, presenting two distinct but converging pathways of AMPK activation in human umbilical vein endothelial cells: one induced by extracellular nucleotides, linked to $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors and dependent on Ca^{2+} and CaMKK, and a second pathway induced by adenosine uptake followed by generation of intracellular AMP and activation of AMPK with LKB1. Our previous results implicated calcium-dependent (via CaMKK-β) and calcium-independent (via LKB1) pathways in the P2-mediated activation of AMPK in podocytes [5]. Nonetheless, considering the protective action of AMPK on glomerular barrier filtration [25], we could assume that maintenance relative to an equilibrium in the functioning of PKA and AMPK is necessary to preserve glomerular function.

Purinergic signaling modulates the cellular response during tissue damage and inflammation, and $P2Y_2$ receptor deficiency is associated with progression of chronic kidney disease. Potthoff et al. [47] showed that $P2Y_2$ receptor knockout mice exhibit increased systolic blood pressure and albuminuria compared to wild-type animals. These authors suggested a significant role for $P2Y_2$ in physiological mechanisms influencing blood pressure and thus endothelial function. The results of many studies have suggested that tissue damage may leads to ROS generation. Low amounts of ROS may function as a second messenger to

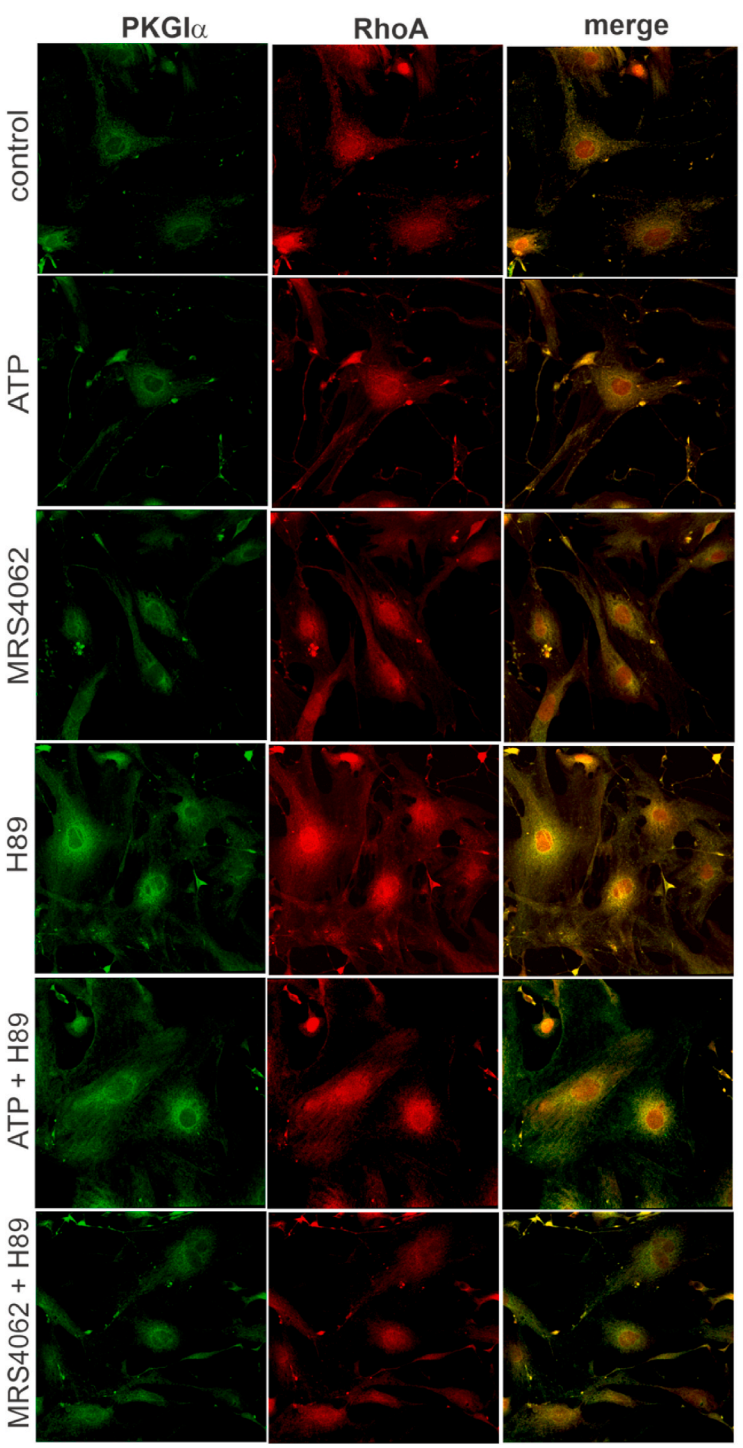
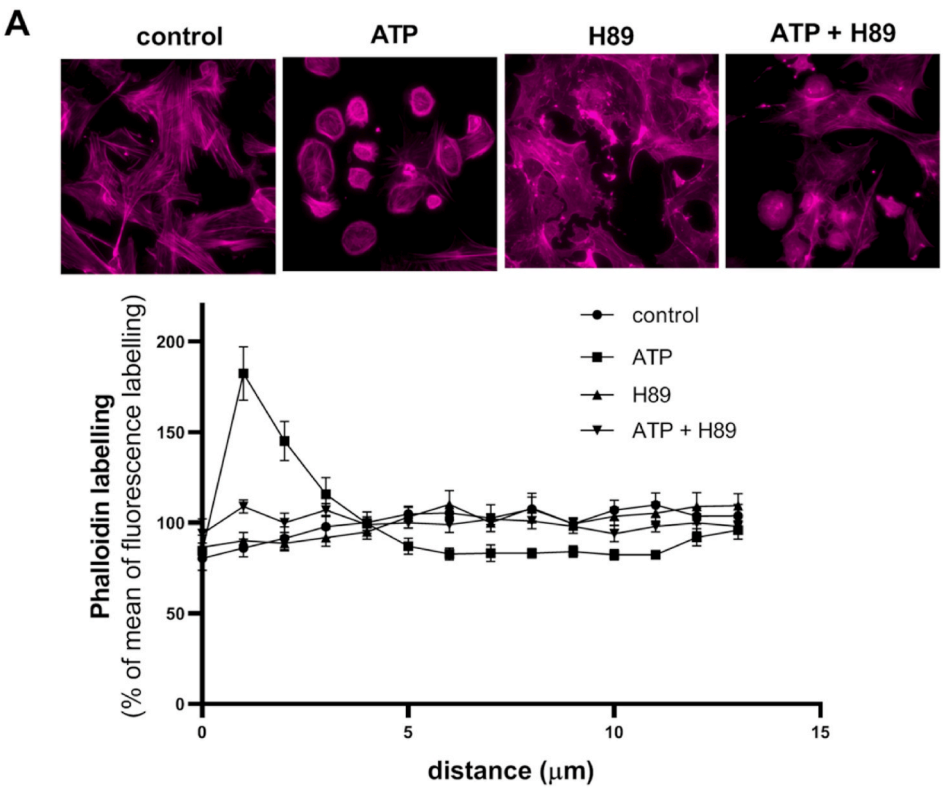


Fig. 8. The effect of P2Y signaling on subcellular localization of PKG and RhoA in cultured rat podocytes. Cells seeded onto coverslips were pre-incubated with H89 (10 μM, 1 h) and incubated for 1 min with 100 μM ATP or 10 μM MRS4062. Cells were immunostained with anti-PKG and anti-RhoA antibodies. Quantitative analysis of protein colocalization was performed with NIS-Elements software (Nikon). The pixel intensities were quantified, and results are given as Pearson's correlation coefficients and colocalization rates (%).Values are reported as the mean ± SEM (n = 10–14). *p < .05 compared to control.

influence redox-sensitive pathways, but higher ROS levels may lead to an imbalance in redox homeostasis and to oxidative damage [48]. In this study, we demonstrated that both extracellular ATP and agonists of $P2Y_1$ and $P2Y_4$ receptors reduced ROS production in podocytes. In previous work, we found that AMPK stimulation reduced activity of NADPH oxidase, a major source of ROS, depending on P2 signaling [29]. Of interest, Förster and Reiser [49] characterized a cytoprotective and antioxidant role of P2Y in oxidative stress in astrocytes. These authors showed that nucleotide treatment directly decreased ROS generation as well as increasing antioxidant defense through stimulation of antioxidant enzymes, and that maintaining basal PKA activity in this process is necessary. In agreement, we postulate that P2 signaling might protect against oxidative injury as a result of the activation of PKA and AMPK.

In contrast, Roshanravan and Dryer [50] found the opposite effect on production ROS after extracellular ATP stimulation. These authors indicated that ATP provokes Ca^{2+} mobilization through TRPC6 activation and subsequent generation of ROS in podocytes. Sustained exposure to extracellular ATP is recognized as causing an oxidative imbalance, but our studies address only the short-term effect of nucleotides on podocyte function. We recently identified a protective role for metformin in TRPC6 channel expression in hyperglycemia mediated by AMPK, presumably associated with suppression of NADPH oxidase activity [26].



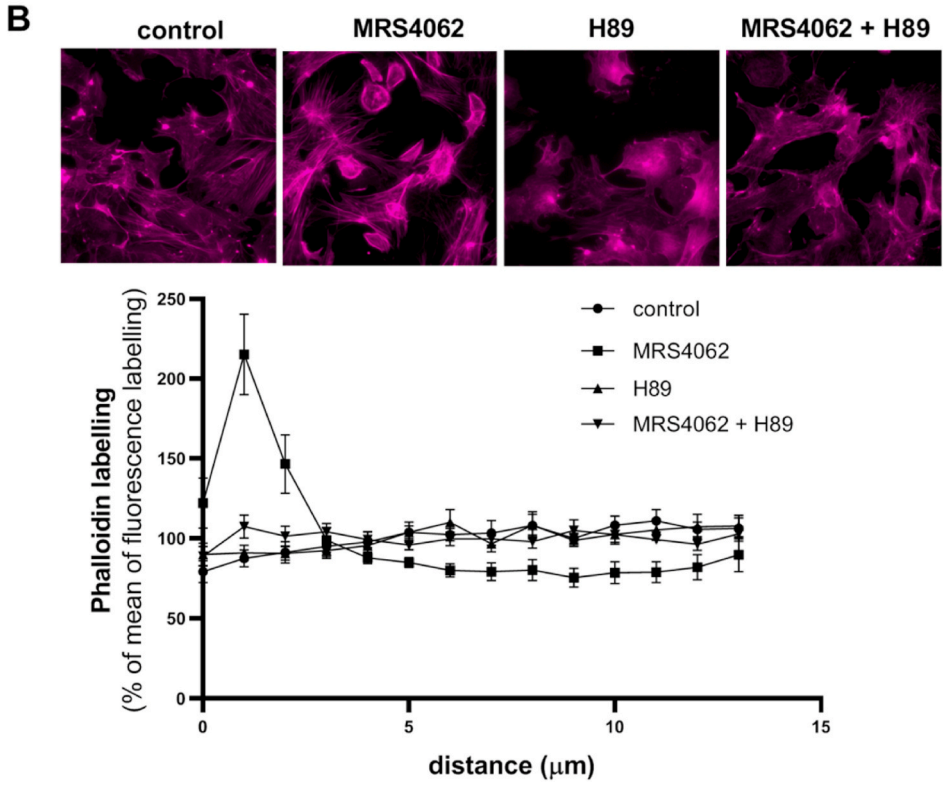


Fig. 9. The effect of inhibition of PKA on remodeling of F-actin cytoskeleton in the presence ATP (A) and MRS4062 (B) in podocytes. Cells were grown on coverslips, pre-incubated with H89 (10 μM, 1 h), and incubated for 1 min with 100 μM ATP (A) or 10 μM MRS4062 (B). The F-actin network was labeled with isothiocyanate phalloidin and visualized with fluorescence microscopy. The digitized fluorescence images of the F-actin network were used to generate the mean intensity and the fluorescence intensity profiles (from the basal membrane to the nucleus) with Cellsens image software. Values are presented as the mean ± SEM (n = 12–14).

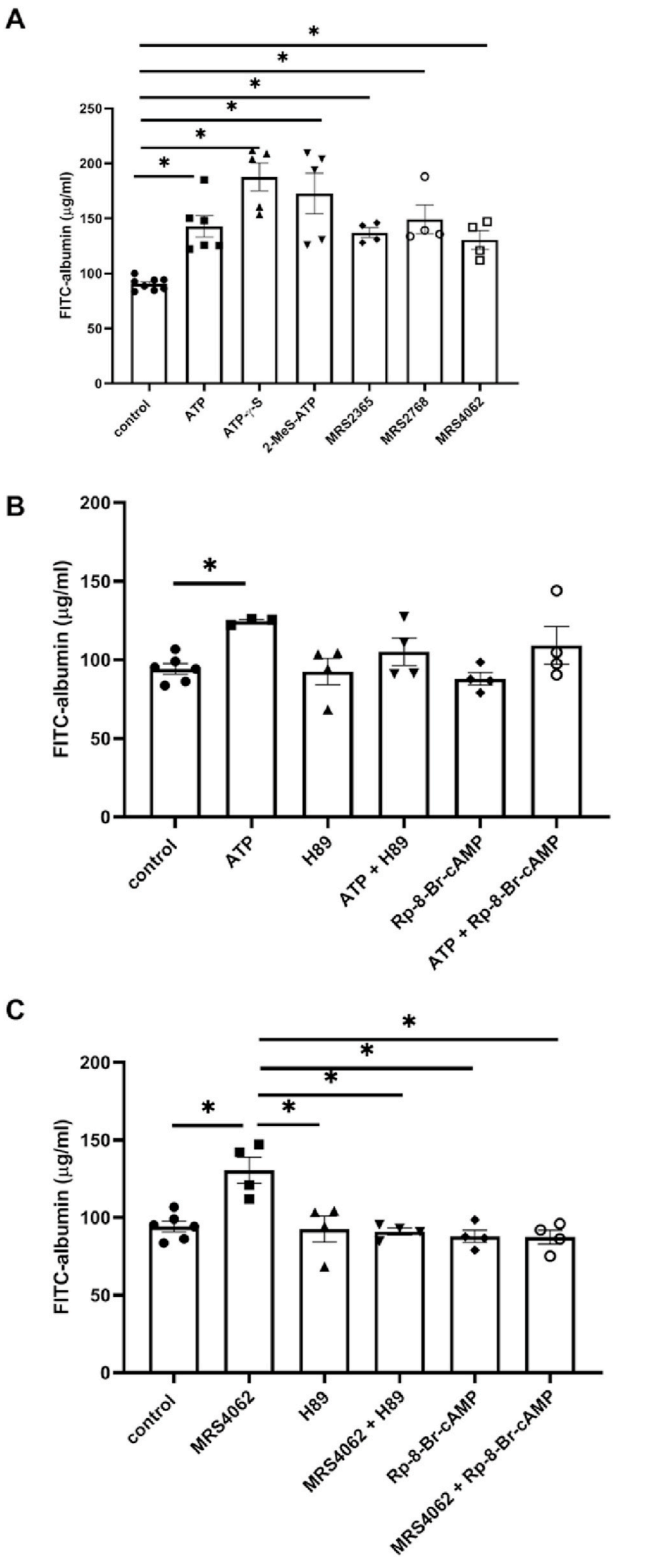


Fig. 10. (A) Effects of P2 signaling on podocyte permeability to albumin. The influence of PKA inhibition on podocyte permeability in the presence of ATP (B) or MRS4062 (C). Transepithelial permeability to albumin was evaluated by measuring the diffusion of FITC-labeled BSA across the podocytes monolayers. Podocytes were incubated with the indicated P2 ligands (A) or preincubated with H-89 or Rp-8-Br-cAMP (10 μM, 1 h) and incubated with 100 μM ATP (B) or 10 μM MRS4062 (C). Values are the mean ± SEM (n = 3–8). *p < .05 compared to control.

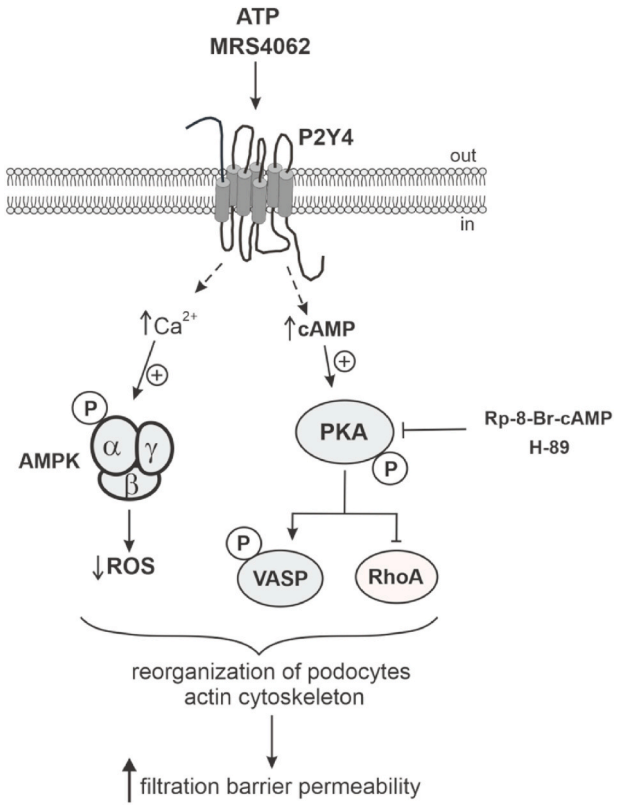


Fig. 11. A proposed mechanism of P2Y-induced regulation of podocyte cytoskeleton organization and filtration barrier permeability involving pleiotropic effects on AMPK and cAMP–PKA signaling pathways.

Therefore, it seems to be possible that under pathological conditions, TRPC6-induced production of ROS is counterbalanced by PKA and AMPK, which suppress ROS generation. This proposed mechanism requires further investigation.

5. Conclusions

In summary, the results of the present study offer evidence supporting a role for purinergic signaling in regulating glomerular filtration through podocyte cytoskeleton remodeling. We propose P2-induced mechanisms of glomerular protection involving PKA activation, leading to regulation of RhoA signaling, rearrangement of actin microfilaments, and restoration of energy homeostasis through AMPK activation, maintaining the oxidative balance through decreased ROS generation.

Funding

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10.3. Kopia pracy poglądowej

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Review

AMPK signalling: Implications for podocyte biology in diabetic nephropathy

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Diabetic nephropathy is a major long-term complication of diabetes mellitus and one of the most common causes of end-stage renal disease. Thickening of the glomerular basement membrane, glomerular cell hypertrophy and podocyte loss are among the main pathological changes that occur during diabetic nephropathy, resulting in proteinuria. Injury to podocytes, which are a crucial component of the glomerular filtration barrier, seems to play a key role in the development of diabetic nephropathy. Recent studies have suggested that dysregulation of AMP-activated kinase protein, which is an essential cellular energy sensor, may play a fundamental role in this process. The purpose of this review is to highlight the molecular mechanisms associated with AMP-activated protein kinase (AMPK) in podocytes that are involved in the pathogenesis of diabetic nephropathy.

Introduction

Diabetes mellitus, which describes a group of metabolic disorders characterised by hyperglycemia and usually resulting from insufficient production of insulin (type 1 diabetes) or an ineffective cellular response to insulin (type 2 diabetes), is a major health problem worldwide. According to the International Diabetes Federation, in 2017 the global prevalence of diabetes was more than 425 million people, and it will increase to 629 million by 2045. Furthermore, the global diabetes-related expenditure in 2017 was estimated to be 727 billion US dollars (International Diabetes Federation, *Diabetes Atlas*. 8th ed.). Diabetic nephropathy (DN), one of the most prevalent chronic complications of diabetes, affects more than 40% of people with diabetes and remains the leading cause of end-stage renal disease in most countries [Reddy et al., 2013; Tuttle et al., 2014]. High mortality associated with diabetic kidney disease has been an increasing problem globally, and searching for a new diagnostic and therapeutic strategy for early prevention and treatment of DN is a challenge for modern science and future medicine.

Early stage DN is characterised by glomerular hyperfiltration and microalbuminuria. As the disease progresses, renal function deteriorates with severe proteinuria, declining glomerular filtration rate and interstitial fibrosis. Albuminuria arises from impairment of the glomerular filtration barrier, resulting in an increase in its permeability and protein leakage into the urine. The pathogenesis of DN involves an interaction between hemodynamic and metabolic factors. Chronic hyperglycemia, hyperinsulinemia, insulin resistance and hyperlipidemia contribute to metabolic imbalance and DN initiation [Reidy et al., 2014]. Disturbed glucose and lipid metabolism initiates several cellular events and activation of signalling

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Key words: AMP-activated protein kinase, Diabetic nephropathy, Glomerular filtration barrier, Podocyte.

Abbreviations: 4E-BP1, 4E-binding protein 1; ACC, acetyl-Co carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; AID, auto-inhibitory domain; AMPK, AMP-activated protein kinase; APPL1, adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1; CaMKKβ, Ca^{++}/calmodulin-dependent protein kinase kinase β; CBS1-4, cystathionine β-synthase repeats 1–4; CPT-1, carnitine palmitoyltransferase-1; DN, diabetic nephropathy; eEF2, eukaryotic elongation factor 2; eEF2 kinase, eukaryotic elongation factor 2 kinase; eIF4E, eukaryotic initiation factor 4E; FFA, free fatty acid; FPs, foot processes; GBD, glycogen binding domain; GBM, glomerular basement membrane; GEC, glomerular epithelial cell; GLUT1, glucose transporter 1; GLUT4, glucose transporter 4; LKB1, tumour-suppressor liver kinase B1; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex-1; p70S6K, p70S6 kinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PTEN, phosphatase and tensin homolog; Rheb, Ras homolog enriched in brain; ROS, reactive oxygen species; SIRT1, deacetylase silence information regulator T1; TAK1, transforming growth factor β-activated kinase; TSC, tuberous sclerosis complex; ZMP, 5-aminoirnidazole-4-carboxamide ribonucleoside monophosphate; ZO-1, zona occludens-1; α-CTD, C-terminal domain of α subunit; β-CTD, C-terminal domain of β subunit.

pathways, which promote structural and functional changes in the kidneys. The key histological and pathological hallmarks of DN include glomerular cell proliferation and hypertrophy, thickening of the glomerular basement membrane (GBM), mesangial expansion and tubulointerstitial fibrosis due to accumulation of extracellular matrix proteins, such as collagens and fibronectin and podocyte loss and foot process effacement [Reddy et al., 2013; Dai et al., 2017].

Podocytes are critical for normal glomerular filtration barrier function

The kidney ensures maintenance of body homeostasis via selective plasma ultrafiltration, which occurs in a highly specialised structure of the nephron, the kidney glomerulus. The glomerulus, which constitutes a capillary network, is composed of three cell types: endothelial cells at the inside of the capillary, podocytes on the outside of the capillary and mesangial cells supporting the capillary loops. Together with the GBM, the endothelium and podocytes form the filtration barrier [Ha, 2013]. Mature podocytes can be divided into three structurally and functionally different segments: cell body, major processes and foot processes. Podocytes wrap the glomerular capillaries in such a way that foot processes (FPs) are attached to the GBM, and cell bodies and major processes float freely in the filtrate in Bowman's space. FPs are anchored to the GBM by transmembrane cell receptors such as integrins and dystroglycans. The foot processes of neighbouring podocytes are interconnected by a specialised cell–cell junction of the podocyte, leaving the filtration slits between them, known as the slit diaphragm. The glomerular filtrate is sieved through the fenestrated endothelial cells, the basement membrane, and finally through the slit diaphragm, which serves as a size-selective barrier. The slit diaphragm constitutes a signalling platform that contains a protein complex of nephrin, P-cadherin, NEPH1, podocin, which play a major role in maintaining the structural and functional integrity of the glomerular filtration barrier [Asanuma and Mundel, 2003]. The slit diaphragm is linked to the actin-based cytoskeleton, located in particular parts of podocytes, by adaptor proteins including, zonula occludens-1 (ZO-1), CD2 associated protein (CD2AP) and Nck. The major components of the cytoskeleton are F-actin, myosin and α-actinin in foot processes (Figure 1).

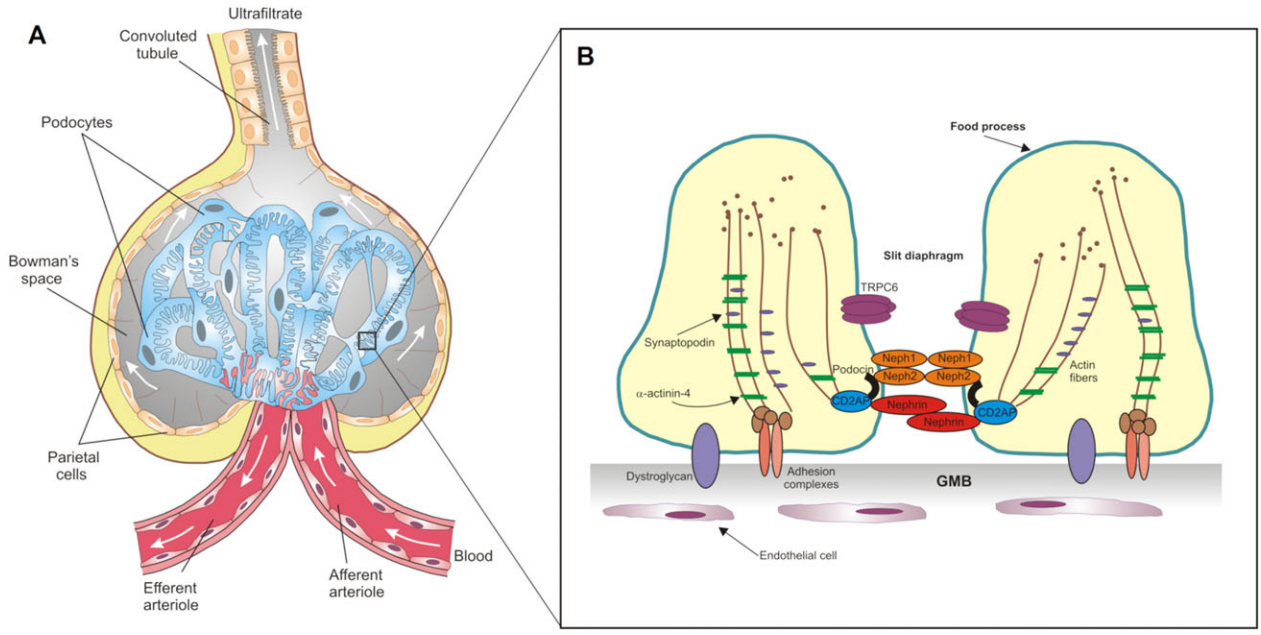
Podocytes express receptors for vasoactive factors, such as angiotensin II, natriuretic peptides or prostaglandins. Therefore, foot processes may regulate cell contractility and counteract the force of filtration, the hydrostatic pressure and modify the properties of glomerular capillaries and the filtration surface area [Pavenstädt, 2000]. Podocytes are highly differentiated postmitotic cells that have a limited capacity to divide [Lasagni et al., 2013]. They are the target of injury in many glomerular diseases, including DN. A number of studies have demonstrated that a disturbance in the metabolic balance and hemodynamic changes in diabetes contribute to the decline in the selective filtering action of the glomerulus, podocyte damage or loss. A decreasing number of podocytes has been observed in patients with diabetes. The mechanism of podocytopenia includes apoptosis, detachment from the GBM or lack of cells to proliferate [Mundel and Shankland, 2002]. Due to their inability to replicate, residual podocytes seem to use different mechanisms to compensate for the cell deficit. The surviving cells undergo changes in size and shape [Reidy et al., 2014]. Increased cell dimensions due to hypertrophy has been observed, as well as foot process effacement as a result of retraction, widening, shortening and simplification of the interdigitating foot processes [Li et al., 2007]. Multiple lines of evidence have assumed that podocyte injury in DN is induced by multiple factors, including mechanical stress, inflammatory reaction, oxidative stress, transforming growth factor beta (TGF-β1) induction, renin angiotensin aldosterone system activation and advanced glycation end product (AGE) accumulation [Dai et al., 2017]. Furthermore, disorders in multiple renal signalling pathways, such as AMP-activated protein kinase (AMPK), have been implicated in the progression of diabetic kidney disease and proteinuria.

Structure, mechanism of action and physiological function of AMPK

AMPK belongs to the serine-threonine protein kinase family. AMPK is heterotrimeric complex consisting of a catalytic α subunit and two regulatory subunits, β and γ, which help maintain the stability of the AMPK complex. In mammals, each subunit is

Figure 1 | Structure of the glomeruli (A). Schematic diagram of the glomerular filtration barrier (B).

The glomerulus constitutes a capillary network that is surrounded by Bowman's space to which primary urine flows. Blood enters the capillaries of the glomerulus by a single arteriole called an afferent arteriole and leaves by an efferent arteriole. Podocytes are highly specialised epithelial cells of glomerulus that cover the outside of glomerular capillaries facing the Bowman's space and the ultrafiltrate. **(B)** The glomerular filter consists of three layers: podocyte, GBM and fenestrated endothelial cells. The molecular complex of nephrin, podocin, Neph 1–2 and CD2AP forms a specialised junction between podocyte foot processes. α–Actinin-4 cross-links various actin fibres which are stabilised by synaptopodin. Adhesion complex and dystroglycans are required for stabilisation of podocyte interaction with GBM. Transient receptor potential cation channel 6 (TRCP6) is a transmembrane channel that interacts directly with the slit diaphragm proteins: nephrin and podocin and regulates intracellular calcium in podocytes.



present as multiple isoforms ($\alpha1, \alpha2, \beta1, \beta2, \gamma1, \gamma2, \gamma3$) encoded by unconnected genes [Hardie, 2014]. The α subunits have conventional serine/threonine kinase domains containing a conserved threonine residue (Thr-172), the phosphorylation of which by upstream kinases is absolutely required for their activity [Hardie, 2008]. Moreover, in the middle region of the α subunit is an autoinhibitory domain that, in the absence of AMP, associates with the kinase domain and blocks the active site. The β subunit contains two conservative domains; one comprises a glycogen-binding site and the other binds with the α and γ subunits and stabilises the heterotrimer AMPK [Hallows et al., 2010]. The γ subunits contain sites for allosteric regulation by AMP/ATP [Russo et al., 2013] (Figure 2).

AMPK is an energy status sensor that maintains cellular and whole-body energy homeostasis. AMPK is activated in response to increasing concentrations of AMP and an increased AMP/ATP ratio under conditions, such as metabolic stress, hypoglycemia, hypoxia, ischemia and exercise, in which consumption of ATP is enhanced. AMPK activation can be regulated through either allosteric activation by AMP or stimulation by upstream kinases, such as the tumour-suppressor liver kinase B1 (LKB1) [Woods et al., 2003], Ca^{++}/calmodulin-dependent protein kinase kinase β (CaMKKβ) [Hawley et al., 2005] and transforming growth factor β-activated kinase (TAK1) [Momcilovic et al., 2006]. Anabolic energy-consuming pathways are inhibited as a result of AMPK activation, whereas catabolic processes generating ATP are stimulated (Figure 3).

AMPK plays a crucial role in maintaining glucose homeostasis, and its activation contributes to glucose transport in a similar way as insulin. AMPK

Figure 2 | Functional domains of AMP-activated protein kinase (AMPK) subunits

The α subunit contains conventional serine/threonine kinase domain which is followed by the auto-inhibitory domain (AID). The β subunit contains two conservative domains; one comprises a glycogen-binding site (GBD). The C-terminal domain of β subunit (β-CTD) works as a scaffold to interact with the α-CTD and the N-terminal of γ subunit to stabilise the heterotrimer. The γ subunits contain sites for allosteric regulation by AMP/ATP, composed of four tandem repeats of sequence [cystathionine β-synthase repeats 1–4 (CBS1–CBS4)] called a CBS motif. Site 4 to have a tightly bound AMP, whereas Sites 1 and 3 may competitively bind AMP, ADP, or ATP.

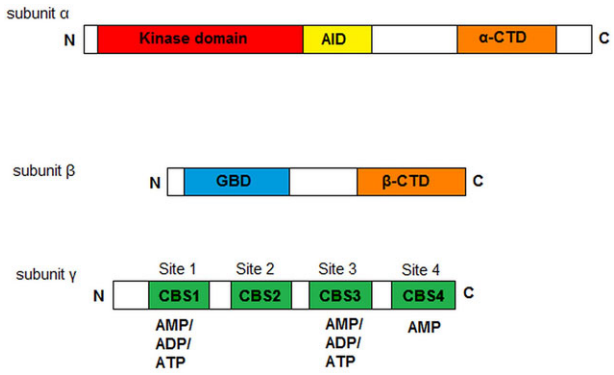
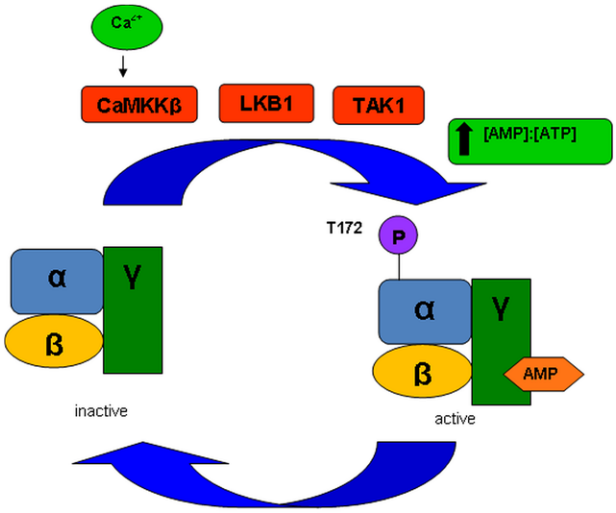


Figure 3 | AMPK regulation

AMPK is activated by phosphorylation at Thr 172, catalysed by three upstream kinases: LKB1, TAK1 and CaMKKβ, which is activated by a rise in cytosol Ca^{2+}. AMPK is activated when AMP/ATP ratio increases. Phosphorylation is stimulated by conformational changes triggered by binding of AMP to the γ subunit of AMPK.



stimulates glucose uptake in skeletal muscle cells through the translocation of glucose transporter 4 (GLUT4) to the cell membrane [Kurth-Kraczek et al., 1999]. In addition, AMPK reduces hepatic gluconeogenesis and glycogen synthesis in skeletal muscles [Lage et al., 2008]. Clinical and experimental data suggest that AMPK is an essential regulator of lipid metabolism. AMPK stimulates fatty acid oxidation in the muscles and liver and diminishes cholesterol and triglyceride synthesis in the liver [Schimmack et al., 2006]. AMPK has been demonstrated to be an important cellular mediator of the metabolic effects of hormones released by peripheral tissues, such as insulin, leptin, adiponectin and resistin, which play a crucial role in metabolism and insulin sensitivity [Lage et al., 2008].

AMPK expression in the kidney

AMPK is expressed in a number of tissues, including the kidney, skeletal muscle, adipose tissue, liver and hypothalamus of the brain [Kim and Park, 2016]. The localisation of AMPK subunits varies, and their expression is tissue specific [Liu and Jiang, 2013]. In the kidney, the $\alpha 1$ subunit is the predominant catalytic isoform, though the $\alpha 2$ subunit is also detectable. In the rat kidney, $\beta 2$ is prevalent, though investigators have found $\beta 1$ to be predominant in the mouse kidney. Both the $\gamma 1$ and $\gamma 2$ subunits are expressed at similar levels in the kidney [Hallows et al., 2010]. AMPK is highly expressed in renal cells, including mesangial cells, glomerular endothelial cells and podocytes [Cammisotto and Bendayan, 2008].

AMPK in diabetes

Given the role of AMPK in regulating energy balance, this kinase occupies a central position in the field of research concerning obesity, diabetes and metabolic syndrome. Accumulating evidence suggests an association between a defect in the AMPK signalling cascade, disorders in metabolic homeostasis and the development of insulin resistance and type 2 diabetes [Ruderman et al., 2013]. Many investigators have indicated diminished AMPK activity in adipose tissue in insulin-resistant humans [Xu et al., 2012] and skeletal muscle from patients with obesity and type 2 diabetes [Bandyopadhyay et al., 2006]. A

number of studies have shown that pharmacological activation of AMPK improves glucose homeostasis, the lipid profile and insulin sensitivity [Amador-Licona et al., 2000], which make this protein kinase an attractive target in the treatment of conditions related to hyperglycemia and insulin resistance.

In experimental models of diabetes, AMPK activity appears to be reduced in the kidney [Lee et al., 2007)]. Several lines of evidence have demonstrated reduced AMPK phosphorylation and activity in glomerular epithelial cells in diabetic conditions [Lee et al., 2007; Eid et al., 2010; Rogacka et al., 2014, 2018]. Moreover, several lines of evidence indicate that pharmacological activation of AMPK exerts favourable nephroprotective effects. Experiments using hypoglycemic drug (metformin) have demonstrated decreased albuminuria in animal models of type 2 diabetes [Kim et al., 2012] and patients with type 2 diabetes mellitus [Amador-Licona et al., 2000]. Metformin (*N*,*N*-dimethylbiguanide) is the most widely used first-line drug for treatment of type 2 diabetes and its therapeutic effects are mediated by AMPK. Metformin inhibits mitochondrial ATP synthesis by inhibiting complex I of the mitochondrial respiratory chain, leading to an increased AMP/ATP ratio [Owen et al., 2000]. Moreover, the generation of reactive nitrogen species ($ONOO^-$) as a result of mitochondrial respiration inhibition by metformin is believed to be responsible for activation of AMPK [Zou et al., 2004]. One of the hypothesis assumes that metformin might increase AMP through inhibition of AMP deaminase [Ouyang et al., 2011]. Interestingly, there are also studies suggesting that AMPK can be activated by metformin without changes in the AMP/ATP ratio [Fryer et al., 2002]. Recent results show that metformin activates AMPK through promoting the formation of the v-ATPase-Ragulator-AXIN/LKB1-AMPK complex [Zhang et al., 2016].

Role of AMPK in oxidative stress

Oxidative stress has been postulated to be a key component in the initiation and progression of DN. Increased generation of reactive oxygen species (ROS) causes oxidation of lipids, proteins and DNA with subsequent tissue damage. Biological consequences of marked oxidative lesions in cells may be a loss of proliferation potential and initiation of apoptosis, which has been implicated in the pathogenesis of diabetic kidney disease. Hyperglycemia-induced overproduction of ROS causes diminution of the number of podocytes that contribute to the development of DN [Susztak et al., 2006].

Several groups have demonstrated potential antioxidant properties of AMPK. Piwkowska et al. [2010] indicated that activation of AMPK by metformin reduces superoxide anion generation in cultured mouse podocytes under normoglycemic, as well as hyperglycemic, conditions. This effect was mediated through down-regulation of the Nox4 - NAD(P)H oxidase subunit. A similar dependence was shown by Eid et al. [2010], assuming that activated AMPK may have protective properties with regard to oxidant stress, which was demonstrated in the abolition of high glucose-induced NADP(H) oxidase activity and Nox4 expression in mouse podocytes. AMPK and its downstream effector Nox4 have been shown to modulate apoptosis through the phosphorylation and activity regulation of tumour-suppressor protein p53 in diabetic conditions. AMPK agonist (5-aminoimidazole-4-carboxamide ribonucleotide, AICAR) has been shown to ameliorate glomerular injury. AICAR is an adenosine analogue that is taken up into cells by adenosine transporters and converted by intracellular adenosine kinase into monophosphorylated form, ZMP (5-aminoirnidazole-4-carboxamide ribonucleoside monophosphate), which binds to AMPK at the same sites as AMP and mimics its activating effect on AMPK [Corton et al., 1995]. Transgenic type 1 diabetic (OVE26) mice treated with AICAR exhibit alleviation of pathological features, such as renal hypertrophy, GBM thickening, podocyte loss and reduction albumin excretion and normalisation of NADPH-dependent superoxide generation. Therefore, AMPK seems to represent the main pathway responsible for the modulation of NADPH oxidase activity, ROS production and oxidative stress, and may protect against podocyte loss and glomerular injury in DN.

Surprisingly, Dugan et al. [2013] presented a novel view on superoxide generation in diabetes. They indicated reduced mitochondrial ROS production in the glomeruli and podocytes of diabetic mice due to decreased mitochondrial function caused by an inactivation of AMPK. Activation of AMPK has been shown to reverse changes in ROS generation. These data indicate that renal mitochondrial-derived superoxide is not a major source of renal oxidant

stress in DN. As mentioned above, AMPK stimulation decreases the production of cytosolic superoxide anion via an NAD(P)H oxidase-dependent mechanism. Therefore, it seems likely that the AMPK cascade exerts a favourable impact on maintaining oxidative balance. Furthermore, pharmacological AMPK activation not only restores mitochondrial superoxide generation, but also normalises albuminuria and urine hydrogen peroxide (H_2O_2) excretion in a diabetic mouse model [Dugan et al., 2013]. The results mentioned above suggest that AMPK protects from renal failure through the maintenance of an appropriate level of cytosolic and mitochondrial ROS.

Impact of AMPK signalling on mitochondrial function

Considering the central role of the mitochondria in the utilisation and production of energy, mitochondrial dysfunction is considered to impact cellular and whole-body metabolic imbalance. Mitochondria are especially important in metabolically active organs, such as the kidneys. Moreover, dysfunction has been implicated in metabolic disease. Several lines of evidence have shown a relationship between disordered mitochondrial function, hyperglycemia and insulin resistance in diabetes, which is thought to contribute to glomerular disease and podocyte injury [Patti and Corvera, 2010]. A number of studies have supposed a protective role of AMPK signalling in the preservation of mitochondrial function. Dugan et al. [2013] demonstrated that AMPK activation promotes mitochondrial biogenesis via peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) stimulation in the glomeruli and podocytes of diabetic mice. Interestingly, Cai et al. [2016] found that grape seed proanthocyanidin extract, a strong antioxidant, prevents high-glucose-induced mitochondrial dysfunction and apoptosis in podocytes via the AMPK-SIRT1-PGC-1α (SIRT1 silent information regulator T1) pathway. This evidence proposed a novel potential therapeutic target in DN through amelioration of mitochondrial function via an AMPK-mediated effect.

Role of the AMPK–mTORC1 axis in apoptosis and hypertrophy

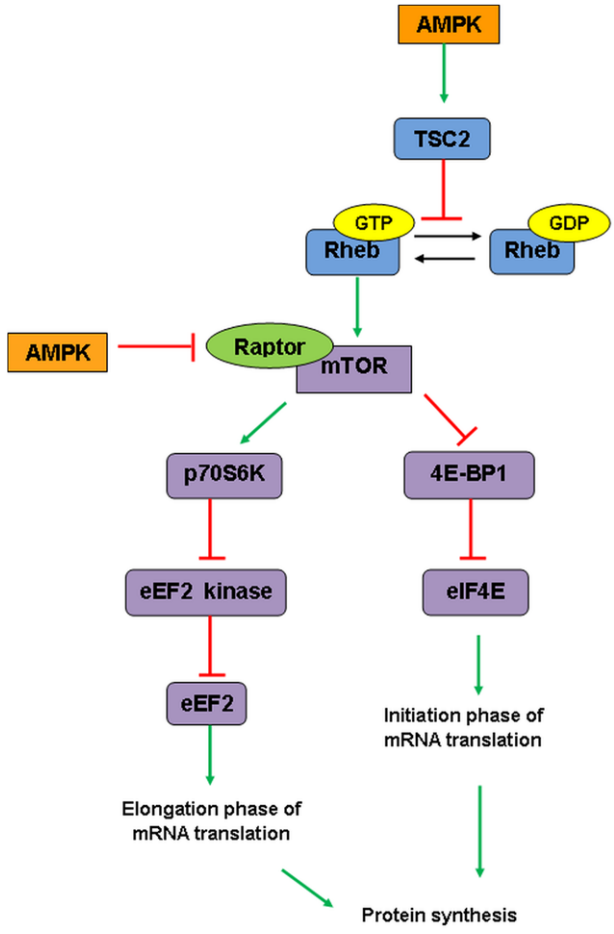
Under diabetic conditions, nutrient excess results in an altered nutritional state and disturbed cellular metabolic homeostasis. Recent research has focused on the role of nutrient-sensing signals, such as AMPK and mammalian target of rapamycin complex-1 (mTORC1), in the progression of diabetic kidney disease [Kume et al., 2014]. Many previous investigators have observed a correlation between the AMPK and mTOR signalling pathways in many cell types and their opposing roles in the control of growth, cell proliferation and metabolism. mTORC1 is a serine/threonine protein kinase and forms two functional complexes, mTORC1 and mTORC2. In addition, mTOR is a downstream pathway regulated by AMPK [Shaw, 2009]. During energy depletion, AMPK suppresses mTORC1 signalling through both direct phosphorylation of the mTORC1 component Raptor and through tuberous sclerosis complex (TSC complex). AMPK phosphorylates TSC2 (also known as tuberin), which manifestations GTPase activating protein properties towards the small G protein Rheb (Ras homolog enriched in brain). TSC2 inactivates Rheb, which has been shown to associate with and directly activate the mTORC1 complex [Inoki et al., 2003; Gwinn et al., 2008]. The AMPK-dependent cascade of events seems to be critical for regulation of mTORC1 signalling pathway, which mediates its downstream effects through the p70S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1) (Figure 4).

In agreement with these reports, Eid et al. [2013] provided evidence that podocyte apoptosis in diabetic conditions is mediated by activation of the mTOR pathway through inactivation of AMPK. Mouse podocytes exposed to high glucose and isolated glomeruli from OVE26 mice exhibit increased phosphorylation of mTOR and p70S6K and decreased tuberin phosphorylation. These effects were reversed in AICAR-treated cells and animals. Langer et al. [2016] demonstrated that metformin-mediated increases in the expression of AMPK and prosurvival factor Akt and decreases in mTOR phosphorylation may be involved in the pro-survival response of podocytes under pro-apoptotic high glucose conditions. Podocyte apoptosis may constitute a characteristic morphological feature of DN that is linked to dysregulation of AMPK signalling and impaired mTOR function.

Multiple lines of research have indicated an association between reduced AMPK activity, upregulation of the mTOR pathway and renal hypertrophy in the

Figure 4 | Schematic of AMPK regulation in mTOR signalling pathway

AMPK suppresses mTORC1 signalling through both direct phosphorylation of the mTORC1 component Raptor and through TSC complex. AMPK phosphorylates TSC2, which manifestations GTPase activating protein properties towards the small G protein Rheb (Ras homolog enriched in brain). TSC2 inactivates Rheb associate with and directly activate the mTORC1 complex. The AMPK-dependent cascade of events seems to be critical for regulation of mTORC1 signalling pathway, which mediates its downstream effects through the p70S6 kinase (p70S6K) and 4E-binding protein 1 (4E-BP1).



diabetic kidney. Accumulation of extracellular matrix proteins and hypertrophy, manifesting as a loss of kidney function in diabetes, is dependent on the increase in protein synthesis, which requires substantial energy expenditure. Therefore, it is not surprising that AMP kinase is considered to be a regulating factor in this process. Consistent with these assumptions, Lee et al. [2007] demonstrated significant inhibition of high-glucose-induced protein synthesis during AMPK activation in glomerular epithelial cells (GECs). The rate of protein synthesis is the result of the regulation of gene expression during mRNA translation. Several key events in the initiation and elongation phase of mRNA translation are under the control of mTORC1. mTORC1-induced phosphorylation of 4E-BP1 and p70S6 kinase results in changes in the phosphorylation of eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 2 kinase (eEF2 kinase) and eukaryotic elongation factor 2 (eEF2), which control the initiation and elongation phase of mRNA translation, respectively [Lee et al., 2012]. AMPK has been recognised to control these processes in GECs through regulation of 4E-BP1 and eEF2 phosphorylation. Moreover, renal hypertrophy has been observed in a streptozotocin-induced model of diabetes and correlated with decreased AMPK phosphorylation in the renal cortex and glomeruli, whereas administration of metformin and AICAR abolished these changes [Lee et al., 2007].

Recently, there has been growing interest in other agents that activate AMPK. Lee et al. [2010] showed that resveratrol, which is known to stimulate AMPK, prevents high glucose-dependent *de novo* protein synthesis and incremental matrix protein fibronectin in GECs. In addition, resveratrol abolished the high glucose effect on the phosphorylation of proteins affecting the initiation and elongation phase of mRNA translation, such as eIF4E, eEF2, eEF2 kinase and p70S6K. Interestingly, this effect was mediated by upstream kinase AMPK – LKB1. The mTOR signalling pathway plays a central role in the regulation of mRNA translation, but the above evidence establishes AMPK interactions with this pathway. Therefore, upregulation of AMPK may ameliorate the injurious effects of high glucose in podocytes and have an inhibitory impact on renal hypertrophy and diabetic kidney disease progression.

Role of AMPK in insulin resistance

There is a growing body of evidence confirming the relationship between glomerular filtration barrier injury and hyperinsulinemia and insulin resistance. Podocytes are insulin-dependent cells and able to absorb glucose via translocation of the glucose transporters GLUT1 and GLUT4 to the plasma

membrane [Coward et al., 2005]. Several groups have demonstrated a loss of podocyte and glomerular insulin signalling in the early stages of diabetic kidney disease [Brosius and Coward, 2014]. The association between insulin signalling and AMPK activity has been proven in insulin-sensitive cells, including podocytes. Numerous studies have found that AMPK mediates insulin-dependent glucose transport in podocytes [Piwkowska et al., 2012; Rogacka et al., 2014]. It has been proposed that development of insulin resistance in podocytes in diabetes can be achieved by interference with AMPK signalling pathway [Rogacka et al., 2014]. Insulin resistance in hyperglycemia has also been demonstrated to correlate with decreased AMPK phosphorylation.

AMPK-SIRT1

Another study has indicated that reduced insulin responsiveness in cultured rat podocytes exposed to high glucose is associated with decreased deacetylase silence information regulator T1 (SIRT1) activity and protein expression and a reduced degree of AMPK phosphorylation [Rogacka et al., 2016]. Increased AMPK activation by the administration of metformin results in enhanced SIRT1 activity in the above-mentioned conditions [Rogacka et al., 2018]. SIRT1 is known to activate AMPK by deacetylating its upstream kinase LKB1 [Lan et al., 2008]. Many studies have presented that SIRT1 exerts similar effects as AMPK on diverse processes, such as cellular metabolism, inflammation and mitochondrial function [Ruderman et al., 2010]. Furthermore, mounting evidence supports a role of SIRT1 in lipid, glucose and energy metabolism and insulin resistance. The renoprotective effects of SIRT1 are found in various models of renal disorders with metabolic impairment, such as DN [Liu et al., 2014]. Thus, the interplay between AMPK and SIRT1 may play a key role in podocyte insulin responsiveness and the maintenance of normal glomerular function.

AMPK-adiponectin

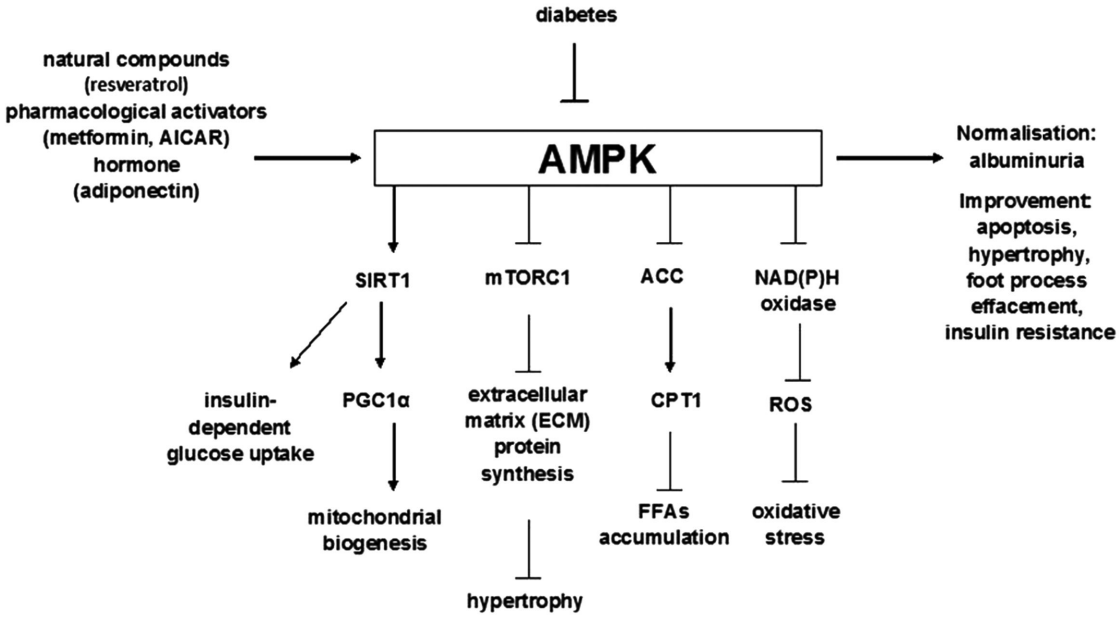
Insulin resistance has been shown to be closely associated with plasma adiponectin levels, which are reduced in obese and diabetic rhesus monkeys [Hotta et al., 2001] and humans with obesity and type 2 diabetes [Weyer et al., 2001]. The insulin-sensitising effect of adiponectin seems to be mediated by the stimulation of glucose utilisation and fatty acid oxidation by the activation of AMPK [Yamauchi et al., 2002]. Moreover, a negative correlation between the albumin excretion rate and plasma adiponectin level in patients with type 2 diabetes has been observed [Ljubic et al., 2015]. Therefore, reduced adiponectin may be a causative factor underlying the deterioration of kidney function in diabetes. Podocytes are thought to be a direct target of adiponectin action. In accordance with this evidence, Sharma et al. [2008] demonstrated that adiponectin administration prevents a reduction of AMPK phosphorylation in high glucose-treated podocytes. These data suggest that adiponectin may improve glucose uptake by stimulating the AMPK pathway. Adiponectin-knockout ($Ad^{-/-}$) diabetic mice treated with adiponectin exhibit normalisation of albuminuria similar to the effect of AICAR. In addition, adiponectin has been shown to reverse foot process effacement in $Ad^{-/-}$ mice. Thus, adiponectin is likely a key regulator of albuminuria, acting through the AMPK pathway.

Role of AMPK in abnormal lipid metabolism

Many studies have indicated a link between DN, disturbed lipid metabolism and renal accumulation of lipids. Moreover, increased triglyceride deposits in the kidney are related to reduced AMPK activity in diabetic kidney disease [Guo and Zhao, 2007]. Suppressed AMPK activity stimulates acetyl-Co carboxylase (ACC) and inhibits carnitine palmitoyltransferase-1 (CPT-1), which in turn extinguishes fatty acid oxidation and leads to accumulation of toxic free fatty acids (FFAs). The increased renal accumulation of lipids results in increased renal abundance of profibrotic factors, leading to glomerulosclerosis and proteinuria in streptozotocin-induced diabetic rats [Guo and Zhao, 2007]. Saturated FFAs elicit insulin resistance in human podocytes [Lennon et al., 2009] and provoke apoptosis in mouse podocytes [Sieber et al., 2010]. Kampe et al. [2014] observed that activation of AMPK prevents lipotoxicity-related podocyte death. These researchers suggested that interference of the AMPK-ACC-CPT-1 pathway plays a key role in eliciting endoplasmic reticulum stress-induced apoptosis due to an increased plasma level of saturated FFAs.

Figure 5 | Summary of AMP-activated protein kinase function in podocytes

The diabetic condition affects inhibitory activity, resulting in morphological and functional disorders of podocytes. Treatment with AMPK activators reduces pathological changes and restores functions.



Impact of AMPK on podocyte actin cytoskeleton

Disorders in slit diaphragm associated proteins are a precisely related to the disorganisation actin cytoskeleton and interference with the podocyte-GBM interaction and subsequent proteinuria. Numerous studies have proved that AMPK may be a protective factor against DN and F-actin derangement. Activation of AMPK in cultured rat podocytes increases activity and phosphorylation the small GTPase- Rac1 that is a major regulator of actin remodelling [Rachubik et al., 2018]. Sharma et al. [2008] indicated reduced podocyte permeability to albumin as a result of AMPK stimulation. AICAR has been demonstrated to restore the translocation of ZO-1 in high glucose-treated podocytes. ZO-1 is a tight junction protein that is highly expressed in the slit diaphragm of the foot process and links slit diaphragm proteins to the actin cytoskeleton [Schnabel et al., 1990]. In line with this finding, another study demonstrated enhanced permeability of the podocyte monolayer in cells exposed to high glucose concentrations and a significant decrease after activation of AMPK by metformin [Rogacka et al., 2018]. Evidence suggests that AMPK inhibition in diabetes may influence the increase in glomerular filtration and reorganisation of the podocyte actin cytoskeleton.

An interesting study by Ji et al. [2015] identified a role for intracellular adaptor protein APPL1 (adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1) that seems to be a pivotal link in adiponectin signalling pathway between receptor AdipoR1 and AMPK and plays an essential role in glucometabolism. These researchers showed that overexpression of APPL1 inhibits high-glucose-induced podocytes apoptosis. Overexpression of APPL1 abolished effect of high glucose on nephrin expression and AMPK and p-AMPK levels.

The activity of AMPK has been closely related to adaptor protein p130Cas that is located in the foot process of podocytes. Ha et al. [2016] found that AICAR and metformin restore distributional and quantitative changes of p130Cas provoked by ANG II in cultured mouse podocytes. p130Cas protein serves as protein linking SD proteins to actin cytoskeleton and therefore may play an important role in actin cytoskeleton reorganisation and preservation the glomerular permeability. P130Cas is likely

to be involved in integrin-mediated signalling and cell adhesion to extracellular matrix. Integrins seems to be key link between foot process of podocytes and GBM. Recently, AMPK has been shown to inhibit integrin activity dependent on tensins [Georgiadou et al., 2017]. Tensin that contains integrin binding region is known as cytoskeleton linker protein and signal transducer [Lo et al., 1997]. Growing body of evidence has shown that tensin2 is a relevant for the maintenance of normal kidney function [Lo et al., 1997; Cho et al., 2006] Moreover, tensin mutation or deficiency in mice have been indicated to alterate of actin remodelling in podocytes, which results in foot process effacement and massive albuminuria [Marusugi et al., 2016; Takahashi et al., 2018]. Tensins are close relatives of phosphatase and tensin homolog (PTEN). Interestingly, PTEN expression has been observed to be down-regulated in the podocytes of diabetic db/db mice and patients with DN. In cultured podocytes, PTEN inhibition caused actin cytoskeletal rearrangement and this reaction was associated with impaired activation of small GTPases Rac1/Cdc42 and RhoA which behave as molecular switches and watch over dynamic regulation of the actin cytoskeleton. Furthermore, increased albumin excretion has been observed in mice treated with the PTEN inhibitor [Lin et al., 2015]. AMPK and PTEN participate together in controlling insulin sensitivity in podocytes [Rogacka et al., 2014], in turn insulin signalling directs actin remodelling in order to glucose uptake [Piwkowska et al., 2015]. The current understanding of the AMPK as principal modulator of cytoskeleton organisation brings new opportunities and hopes in DN therapy.

In summary, AMPK protein has been shown to play an essential role in maintaining metabolic homeostasis in multiple cellular mechanisms in glomerular epithelial cells and the preservation of normal renal function (Figure 5).

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11. Pisemne oświadczenia autorów prac tworzących zbiór

11.1. Oświadczenia kandydata określające jego indywiduwalny wkład w powstanie każdej z prac tworzących zbiór

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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Kreft E, Angielski S, Piwkowska A; Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions, Biochim Biophys *Acta Mol Basis Dis*, 2020 1;1866(3):165610" mój udział polegał na uczestniczeniu w koncepcji projektu, wykonaniu części doświadczeń, obliczeń i analizy statystycznej wyników, a także interpretacji wyników oraz współudziale w pisaniu manuskryptu, w tym opisu metod i wstępu.

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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A, Extracellular ATP modulates podocyte function through P2Y purinergic receptors and pleiotropic effects on AMPK and cAMP/PKA signaling pathways, *Arch Biochem* Biophys, 2020 Nov 30;695:108649. doi: 10.1016/j.abb.2020.108649. Epub 2020 Oct 22." mój udział polegał na uczestniczeniu w koncepcji projektu, zaplanowaniu oraz wykonaniu większości doświadczeń, wykonaniu obliczeń i analizy statystycznej wyników, interpretacji wyników, przygotowaniu większości rycin, napisaniu manuskryptu, a następnie dyskusji z recenzentami i edycji ostatecznej wersji manuskryptu.

Maria Szrejder

Gdańsk, 08.12.2020

mgr Maria Szrejder
Pracownia Molekularnej i Komórkowej Nefrologii
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im. M Mossakowskiego PAN
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02-106 Warszawa

Oświadczenie

Oświadczam, że w pracy „Szrejder M, Piwkowska A, AMPK signalling: Implications for podocyte biology in diabetic nephropathy, Biol Cell,2019 111(5):109-120." mój udział polegał na stworzeniu koncepcji oraz redakcji manuskryptu, a następnie dyskusji z recenzentami i edycji ostatecznej wersji manuskryptu.

Maria Szrejder

11.2. Oświadczenia pozostałych współautorów prac tworzących zbiór, w których wyrażają oni zgodę na wykorzystanie wspólnych publikacji w przewodzie doktorskim kandydata oraz określają swój indywidualny wkład w ich powstanie

Gdańsk, 08.12.2020

mgr Patrycja Rachubik
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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Kreft E, Angielski S, Piwkowska A; Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions, Biochim Biophys *Acta Mol Basis Dis*, 2020 1;1866(3):165610" mój udział polegał na sprawowaniu opieki nad zwierzętami wykorzystywanymi do doświadczeń, a także przeprowadzeniu eksperymentów na zwierzętach dotyczących wyidukowania cukrzycy przy użyciu streptozotocyny, pomiaru stężenia glukozy we krwi oraz albuminy w moczu, a także pomiaru przepuszczalności dla albuminy na izolowanych kłębuszków nerkowych. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Marii Szrejder.

Patrycja Rachubik

Gdańsk, 08.12.2020

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Gdańsk, 08.12.2020

dr n. med. Irena Audzeyenka
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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Kreft E, Angielski S, Piwkowska A; Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions, Biochim Biophys *Acta Mol Basis Dis*, 2020 1;1866(3):165610" mój udział polegał na wykonaniu części doświadczeń, które obejmowały izolację mRNA oraz zaprojektowanie i przeprowadzenie reakcji real-time PCR. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Marii Szrejder.

Irena Audzeyenka

Gdańsk, 08.12.2020

dr hab. n. med. inż. Agnieszka Piwkowska
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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Kreft E , Angielski S, Piwkowska A; Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions, Biochim Biophys Acta Mol Basis Dis, 2020 1;1866(3):165610" mój udział polegał na sformułowaniu hipotezy badawczej, zaplanowaniu i kierowaniu projektem badawczym, wykonaniu części doświadczeń, opracowaniu analizy statystycznej uzyskanych wyników oraz ich interpretacji, a także przygotowaniu manuskryptu oraz edycji jego ostatecznej wersji. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Marii Szrejder.

Piwkowska

Gdańsk, 08.12.2020

mgr Maria Szrejder
Pracownia Molekularnej i Komórkowej Nefrologii
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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D , Audzeyenka I , Rychłowski M , Kreft E , Angielski S , Piwkowska A, Metformin reduces TRPC6 expression through AMPK activation and modulates cytoskeleton dynamics in podocytes under diabetic conditions, Biochim Biophys Acta Mol Basis Dis, 2020 1;1866(3):165610" udział pozostałych autorów polegał na:

	Imię i nazwisko	Udział
1.	dr Michał Rychłowski Zakład Biologii Molekularnej Wirusów Międzyuczelniany Wydział Biotechnologii UG i GUMed Ul. Abrahama 58 80-307 Gdańsk	Analiza preparatów barwionych immunofluorescencyjnie pod mikroskopem konfokalnym.
2.	dr n. farm. Ewelina Kreft Zakład Chemii Klinicznej Gdański Uniwersytet Medyczny Ul. Dębinki 7 80-211 Gdańsk	Ocena klirensu radioaktywnej inuliny oraz przepływu krwi przez korę nerki metodą laser-Doppler.
3.	prof. dr hab. med. Stefan Angielski Pracownia Molekularnej i Komórkowej Nefrologii Instytut Medycyny Doświadczalnej i Klinicznej im. M Mossakowskiego PAN Ul. A. Pawińskiego 5 02-106 Warszawa	Uczestniczenie w koncepcji projektu oraz nadzór merytoryczny nad wykonaniem projektu, końcowa akceptacja manuskryptu.

Maria Szrejder

Gdańsk, 08.12.2020

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Ul. A. Pawińskiego 5
02-106 Warszawa

Oświadczenie

Oświadczam, że w pracy „Szrejder M, Rachubik P, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A, Extracellular ATP modulates podocyte function through P2Y purinergic receptors and pleiotropic effects on AMPK and cAMP/PKA signaling pathways, *Arch Biochem* Biophys, 2020 Nov 30;695:108649. doi: 10.1016/j.abb.2020.108649. Epub 2020 Oct 22." mój udział polegał na formatowaniu, edycji i ostatecznej akceptacji manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Marii Szrejder.

Patrycja Rachubik

Gdańsk, 08.12.2020

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Ul. A. Pawińskiego 5
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Gdańsk, 08.12.2020

dr n. med. Irena Audzeyenka
Pracownia Molekularnej i Komórkowej Nefrologii
Instytut Medycyny Doświadczalnej i Klinicznej
im. M Mossakowskiego PAN
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02-106 Warszawa

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Irena Audzeyenka

Gdańsk, 08.12.2020

dr hab. n. med. inż. Agnieszka Piwkowska
Pracownia Molekularnej i Komórkowej Nefrologii
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Ul. A. Pawińskiego 5
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Piwkowska

Gdańsk, 08.12.2020

mgr Maria Szrejder
Pracownia Molekularnej i Komórkowej Nefrologii
Instytut Medycyny Doświadczalnej i Klinicznej
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2.	prof. dr hab. med. Stefan Angielski Pracownia Molekularnej i Komórkowej Nefrologii Instytut Medycyny Doświadczalnej i Klinicznej im. M Mossakowskiego PAN Ul. A. Pawińskiego 5 02-106 Warszawa	Uczestniczenie w koncepcji projektu oraz nadzór merytoryczny nad wykonaniem projektu.

Maria Szrejder

Gdańsk, 08.12.2020

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Oświadczenie

Oświadczam, że w pracy „Szrejder M, Piwkowska A, AMPK signalling: Implications for podocyte biology in diabetic nephropathy, *Biol Cell*, 2019 111(5):109-120." mój udział polegał na nadzorowaniu przygotowania manuskryptu oraz redakcji końcowej manuskryptu. Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Marii Szrejder.

Piwkowska