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Rola kinazy białkowej PKGIα i kanału jonowego TRPC6 w regulacji funkcji podocytów w warunkach fizjologicznych i wybranych stanach patofizjologicznych

The Role of Protein Kinase G Type Iα and TRPC6 Channel in Regulating Podocyte Function in Physiological Conditions and Selected Pathophysiological States

Rozprawa na stopień naukowy doktora
w dziedzinie nauk medycznych i nauk o zdrowiu w dyscyplinie nauki medyczne

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2. Rachubik P, Szrejder M, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A. 2018. The TRPC6-AMPK pathway is involved in insulin-dependent cytoskeleton reorganization and glucose uptake in cultured rat podocytes. *Cell Physiol Biochem,* 51(1): 393-410; IF_5=4,354 (2017)

3. Rachubik P, Szrejder M, Audzeyenka I, Rogacka D, Rychłowski M, Angielski S, Piwkowska A. 2020. The PKGIα/VASP pathway is involved in insulin- and high glucose-dependent regulation of albumin permeability in cultured rat podocytes. *J Biochem,* 168(6): 575-588; IF_5=2,381 (2019)

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Wykaz skrótów stosowanych w rozprawie doktorskiej

Akt – (*ang. protein kinase B*), kinaza białkowa B

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

BK_{Ca} – *(ang. large-conductance Ca^{2+}-activated potassium channel)*, kanał potasowy aktywowany przez jony Ca^{2+} o dużym przewodnictwie

BSA – (*ang. bovine serum albumin*), surowicza albumina wołowa

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

CaMKKβ – (*ang. calcium/calmodulin-dependent protein kinase kinase β*), kinaza kinaz Ca^{2+}/kalmodulinozależnych β

CC – (*ang. compound C*), związek C, inhibitor AMPK

DKD – (*ang. diabetic kidney disease*), cukrzycowa choroba nerek

EDTA – (*ang. ethylenediaminetetraacetic acid*), kwas etylenodiaminotetraoctowy, kwas wersenowy

FBS - (*ang. fetal bovine serum*), płodowa surowica bydlęca

GLUT4 – (*ang. glucose transporter type 4*), transporter glukozowy typu 4

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

INS – (*ang. insulin*), insulina

IR – (*ang. insulin receptor*), receptor insulinowy

IRS-1 – (*insulin receptor substrate 1*), substrat receptora insulinowego 1

MLC – (*ang. myosin light chain*), łańcuch lekki miozyny

MLCK - (*ang. myosin light chain kinase*), kinaza łańcuchów lekkich miozyny

MLCP - (*ang. myosin light chain phosphatase*), fosfataza łańcuchów lekkich miozyny

mRNA – (*ang. messenger ribonucleic acid*), informacyjny kwas rybonukleinowy

MTF – (*ang. metformin*), metformina

MYPT1 – (*ang. myosin phosphatase target subunit 1*), docelowa podjednostka 1 fosfatazy miozyny, podjednostka regulatorowa fosfatazy białkowej

NADPH – (*ang. nicotinamide adenine dinucleotide*), dinukleotyd nikotynoamidoadeninowy

NO – *(ang. nitric oxide),* tlenek azotu

NOX4 – (*ang. oxidase NAD(P)H 4*), oksydaza NAD(P)H typu 4

OAG – (*ang. 1-oleoyl-2-acetyl-sn-glycerol*), 1-oleilo-2-acetyloglicerol, analog diacyloglicerolu, aktywator kanału TRPC

P_{alb} – (*ang. albumin permeability*), konwekcyjny współczynnik przepuszczalności dla albuminy liczony dla izolowanych kłębuszków nerkowych

PAK – (*ang. p21-activated kinase*), kinaza aktywowana przez białka p21

PAR1 – (*ang. protease-activated receptor 1*), receptor aktywowany proteazą 1, receptor trombinowy

PBS – (*ang. phosphate-buffered saline*), sól fizjologiczna buforowana fosforanem

PCR – (*ang. polymerase chain reaction*), reakcja łańcuchowa polimerazy

PKGIα – (*ang. protein kinase G type Iα*), kinaza białkowa G typu Iα

ROCK – (*ang. Rho-associated protein kinase*), kinaza białkowa związana z Rho

ROCs – *(ang. receptor-operated channels)*, kanały regulowane przez receptory

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

Rp-8-Br-cGMPS – (*ang. Rp-8-bromoguanosine- 3', 5'- cyclic monophosphorothioate sodium salt*), analog cGMP, inhibitor PKG

RT – *(ang. room temperature)*, temperature pokojowa

SD – (*ang. slit diaphragm*), błona filtracyjna

SDS – (*ang. sodium dodecyl sulfate*), siarczan dodecylu sodu

SDS-PAGE – (*ang. SDS-poliacrylamide gel electrophoresis*), elektroforeza białek w żelu poliakryladmidowym w warunkach denaturujących

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

siRNA – (*ang. small interfering RNA*), małe interferujące RNA

TRP – *(ang. transient receptor potential)*, receptor przejściowego potencjału

TRPA - *(ang. transient receptor potential ankyrin)*, receptor przejściowego potencjału ankirynowy

TRPC6 – (*ang. transient receptor potential canonical type 6*), receptor przejściowego potencjału kanoniczny typu 6

TRPM - *(ang. transient receptor potential)*, receptor przejściowego potencjału melastatynowy

TRPML - *(ang. transient receptor potential)*, receptor przejściowego potencjału mukolipinowy

TRPP - *(ang. transient receptor potential)*, receptor przejściowego potencjału policystynowy

TRPV - *(ang. transient receptor potential)*, receptor przejściowego potencjału waniloidowy

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

8-Br-cGMP – (*ang. 8-bromo-cyclic GMP*), bromowana pochodna cGMP, aktywator PKG

σ_{alb} – (*ang. albumin reflection coefficient*), współczynnik przepuszczalności dla albuminy liczony dla izolowanych kłębuszków nerkowych

$[Ca^{2+}]_i$ – (*ang. intracellular concentration of calcium ions*), wewnątrzkomórkowe stężenie jonów wapnia

1. Streszczenie polskojęzyczne i anglojęzyczne

Rola kinazy białkowej PKGIα i kanału jonowego TRPC6 w regulacji funkcji podocytów w warunkach fizjologicznych i wybranych stanach patofizjologicznych

Najważniejszym elementem kłębuszkowej bariery filtracyjnej są podocyty – komórki wrażliwe na działanie insuliny, regulujące przepływ filtratu przez szczelinę filtracyjną utworzoną przez przeplatające się wypustki stopowate sąsiednich podocytów. Utrata prawidłowej funkcji tych komórek prowadzi do zmian w przepuszczalności kłębuszkowej bariery filtracyjnej. Zwiększona przepuszczalność bariery filtracyjnej skutkuje rozwojem albuminurii, pierwszego klinicznego objawu cukrzycowej choroby nerek (DKD). Molekularne mechanizmy regulujące funkcjonowanie podocytów i przepuszczalność bariery filtracyjnej w cukrzycy wciąż nie są dokładnie poznane.

Jony wapnia (Ca^{2+}) są ważnymi mediatorami homeostazy komórkowej, a ich napływ do komórki jest regulowany m. in. przez kanał jonowy TRPC6. W podocytach, kanał TRPC6 jest zaangażowany w insulinozależną regulację dynamiki szkieletu aktynowego, a tym samym odgrywa istotną rolę w regulacji przepuszczalności bariery filtracyjnej podczas fizjologicznych wahań filtracji kłębuszkowej.

W insulinozależną regulację przepuszczalności kłębuszkowej bariery filtracyjnej zaangażowana jest również kinaza białkowa G typu Iα (PKGIα). Wcześniejsze badania zespołu potwierdzają występowanie zależności pomiędzy aktywacją PKGIα, reorganizacją szkieletu aktynowego, a zwiększoną przepuszczalnością dla albuminy przez warstwę podocytów utworzoną na nylonowej membranie pokrytej kolagenem typu IV.

Rozregulowanie szlaku sygnałowego zależnego od TRPC6 oraz PKGIα wpływa negatywnie na funkcję podocytów, dlatego też istotne jest poznanie szlaków sygnałowych aktywowanych przez te białka. Celem pracy było zbadanie roli PKGIα i TRPC6 w regulacji szlaków sygnałowych kluczowych dla funkcjonowania podocytów zarówno w warunkach fizjologicznych, jak i patofizjologicznych. W cyklu publikacji stanowiących podstawę niniejszej rozprawy doktorskiej zaprezentowano dowody potwierdzające występowanie zależności pomiędzy aktywnością TRPC6, PKGIα, a insulinozależną regulacją przepuszczalności kłębuszkowej bariery filtracyjnej. Badania *in vitro* wykazały, że insulina zwiększa przepuszczalność kłębuszkowej bariery filtracyjnej poprzez aktywację szlaku sygnałowego PKGIα/TRPC6. Ponadto insulina indukuje proces reorganizacji aktyny

w wyniku TRPC6-zależnego napływu jonów Ca^{2+} do komórek podocytarnych hodowanych w warunkach *in vitro*.

TRPC6 odgrywa również kluczową rolę w regulacji insulinozależnego dokomórkowego transportu glukozy w podocytach oraz wpływa na aktywności kinazy białkowej aktywowanej przez AMP (AMPK), głównego regulatora metabolizmu glukozy. Co więcej, aktywacja AMPK i TRPC6 jest niezbędna do pobudzenia szlaku sygnałowego zależnego od białka Rac1, którego aktywność wiąże się z kontrolą dynamiki szkieletu aktynowego i z dokomórkowym transportem glukozy.

Warunki patofizjologiczne, takie jak hiperinsulinemia czy hiperglikemia, zaburzają prawidłowe funkcjonowanie podocytów w wyniku aktywacji szlaku sygnałowego PKGIα/VASP. Wysokie stężenia insuliny lub glukozy zwiększają zarówno ilość białka VASP, jak i PKGIα-zależną fosforylację tego białka w pozycji Ser239, inicjują przebudowę szkieletu aktynowego oraz zwiększają przepuszczalność monowarstwy podocytów dla albuminy w sposób zależny od białka VASP.

Powyższe wyniki świadczą o istotnej roli szlaku sygnałowego zależnego od insuliny oraz sygnalizacji wapniowej w regulacji funkcji podocytów oraz kłębuszków nerkowych, co może przekładać się na funkcjonowanie nerek. Zaburzenia sygnalizacji zależnej od insuliny i TRPC6 mogą znacząco zaburzyć stan energetyczny podocytów, upośledzając ich funkcjonowanie, co z kolei może prowadzić do zmian w przepuszczalności kłębuszkowej bariery filtracyjnej. Otrzymane wyniki badań sugerują również, iż jednym z czynników indukujących rozwój DKD jest aktywacja szlaku sygnałowego PKGIα/VASP, mogąca prowadzić do wzrostu przepuszczalności bariery filtracyjnej.

The Role of Protein Kinase G Type Iα and TRPC6 Channel in Regulating Podocyte Function in Physiological Conditions and Selected Pathophysiological States

Insulin-sensitive podocytes are a key part of glomerular filtration barrier. They control the glomerular filtrate pass across the filtration slit – a structure formed by interdigitating foot processes of podocytes. Loss of podocyte function leads to alternation of glomerular filtration barrier permeability. Increased glomerular barrier permeability results in albuminuria, the first clinical sign of diabetic kidney disease (DKD). Molecular mechanisms that regulate podocyte functioning and glomerular filtration barrier permeability in diabetes are still not fully understood.

Calcium ions (Ca^{2+}) are important mediators of cell homeostasis, and their influx is regulated by ion channels, including TRPC6. In podocytes, TRPC6 is involved in insulin-dependent regulation of the actin cytoskeleton dynamics, and thus in the regulation of glomerular filtration barrier permeability during physiological fluctuations in glomerular filtration.

Protein kinase G type Iα (PKG Iα) is also involved in the regulation of glomerular filtration barrier permeability. Recent studies have confirmed a relationship between PKGIα activation, actin cytoskeleton reorganization and increased albumin permeability across the layer of podocytes that cover type IV collagen-coated nylon membrane.

Dysregulation of the TRPC6 and PKGIα signaling pathways has negative impact on podocyte function. Therefore, it is important to identify signaling pathways that are activated by these proteins. The aim of this paper was to investigate the role of PKGIα and TRPC6 in the regulation of the signaling pathways crucial for podocyte functioning under both physiological and pathophysiological conditions. The present doctoral dissertation is based on a series of publications that have confirmed the relationship between the activities of TRPC6 and PKGIα, and insulin-dependent regulation of glomerular filtration barrier permeability. The conducted *in vitro* research has shown that insulin increases glomerular filtration barrier permeability via PKGIα/TRPC6 signaling pathway activation. Moreover, insulin induces actin reorganization as a result of the TRPC6-dependent Ca^{2+} influx into cultured podocytes.

TRPC6 also plays a crucial role in the regulation of insulin-dependent glucose uptake in podocytes and affects the activity of AMP-activated protein kinase (AMPK), the main regulator of glucose metabolism. Furthermore, activation of both AMPK and TRPC6 is required for triggering Rac1 signaling pathway. The Rac1 signaling is associated with control of actin cytoskeleton dynamics and glucose uptake.

Pathophysiological conditions like hyperinsulinemia or hyperglycemia disturb podocyte functioning through the activation of the PKGIα/VASP signaling pathway. High concentrations of insulin or glucose increase both the amount of VASP and PKGIα-dependent phosphorylation of VASP at Ser239. Moreover, insulin and glucose induce actin cytoskeleton remodeling as well as augment albumin permeability across the podocyte layer in a VASP-dependent manner.

These results clearly demonstrate the significance of the insulin-dependent signaling pathway and calcium signaling in the regulation of the functioning of podocytes and glomeruli, which may affect renal function. Disturbances in the insulin-dependent and TRPC6 signaling may significantly decrease the energy state of podocytes, thus impairing their function, and consequently, it may lead to alternations in glomerular filtration barrier permeability. The results obtained in the course of the present study also suggest that DKD development is induced by the activation of the PKGIα/VASP signaling pathway, which may result in increased glomerular filtration barrier permeability.

2. Oryginalność rozprawy

W ramach niniejszej rozprawy doktorskiej wykazano, że:

- kanał jonowy TRPC6 wpływa na aktywność PKGIα oraz bierze udział w insulinozależnej regulacji przepuszczalności kłębuszkowej bariery filtracyjnej;

- wysokie stężenia insuliny oraz glukozy, poprzez aktywację szlaku sygnałowego PKGIα/VASP, regulują przepuszczalność albuminy przez monowarstwę podocytów;

- TRPC6-zależna aktywacja AMPKα2 ma kluczowe znaczenie w insulinozależnej regulacji dynamiki szkieletu aktynowego podocytów;

- szlak sygnałowy TRPC6/AMPKα2 odgrywa zasadniczą rolę w regulacji insulinozależnego dokomórkowego transportu glukozy w szczurzych podocytach.

3. Wstęp

Cukrzycowa choroba nerek (DKD) jest najczęściej występującym powikłaniem cukrzycy typu 2 charakteryzującym się morfologicznymi oraz czynnościowymi zmianami zachodzącymi w nerkach, wywołanymi bezpośrednio przez przewlekłą hiperglikemię[1]. Pierwszym klinicznym objawem DKD jest albuminuria, rozwijająca się w wyniku uszkodzenia kłębuszkowej bariery filtracyjnej – selektywnej struktury odpowiedzialnej za filtrację osocza[2].

Kłębuszek nerkowy stanowi fundamentalną jednostkę nerki odpowiedzialną za czynności filtracyjne. Sam kłębuszek zbudowany jest z tętniczki doprowadzającej rozdzielającej się na sieć naczyń włosowatych, które zbiegają się w tętniczkę odprowadzającą. Naczynia krwionośne kłębuszka nerkowego otoczone są torebką Bowmana, zaś przestrzenie pomiędzy naczyniami włosowatymi wypełnia mezangium wewnątrzkłębuszkowe[3]. Kłębuszek nerkowy może pełnić swoją funkcję dzięki wspomnianej wcześniej kłębuszkowej barierze filtracyjnej składającej się z komórek śródbłonka naczyń włosowatych, błony podstawnej kłębuszka nerkowego oraz podocytów – komórek ściśle oplatających naczynia włosowate kłębuszka[4].

Podocyty są ostatecznie zróżnicowanymi komórkami nabłonkowymi w obrębie których wyróżnia się trzy strukturalne i funkcjonalne segmenty: ciało komórki, wypustki główne i wypustki stopowate zawierające aparat kurczliwy zbudowany m. in. z filamentów aktynowych, miozyny, winkuliny[5,6]. Pomiędzy sąsiadującymi ze sobą wypustkami stopowatymi znajduje się szczelina filtracyjna, która jest ograniczona najważniejszym elementem bariery filtracyjnej - błoną szczelinową (SD) przez którą przepływa filtrat[7]. SD jest zakotwiczona w podstawo-bocznych regionach wypustek stopowatych łącząc je ze sobą. Struktura ta stanowi platformę sygnalizacyjną komórek podocytarnych regulując ich funkcjonowanie i morfologię[8]. W skład SD wchodzi wiele białek tworzących funkcjonalny kompleks m. in.: nefryna, podocyna, kanał jonowy TRPC6 oraz aktyna[8–10].

Podocyty są komórkami wrażliwymi na działanie insuliny, która odgrywa kluczową rolę w ich prawidłowym funkcjonowaniu. Hormon ten odpowiada za regulację dynamiki filamentów aktynowych w podocytach oraz jest istotnym czynnikiem stojącym na straży integralności kłębuszkowej bariery filtracyjnej[11]. Zasadniczym zadaniem insuliny jest regulacja dokomórkowego transportu glukozy, głównie poprzez zwiększenie translokacji wewnątrzkomórkowych pęcherzyków zawierających transportery glukozowe typu 4 (GLUT4) w kierunku błony komórkowej podocytów[12,13]. W przypadku komórek

podocytarnych z wyindukowaną wysokim stężeniem glukozy insulinoopornością dochodzi do zniesienia efektu insuliny na dokomórkowy transport glukozy[14], co może negatywnie wpływać na pełnione przez nie funkcje.

Badania wykazały, że zaburzenia sygnalizacji insulinowej w mysich podocytach są związane z rozwojem albuminurii, której towarzyszą zmiany charakterystyczne dla chorych na DKD[11]. Oprócz tego, eksperymenty przeprowadzone w naszym zespole dowodzą, że insulina inicjuje proces reorganizacji szkieletu aktynowego, co wiąże się ze zwiększoną przepuszczalnością albuminy przez monowarstwę podocytów[15,16]. Podobny efekt zaobserwowano w szczurzych kłębuszkach nerkowych inkubowanych z insuliną oraz kłębuszkach izolowanych z nerek otyłych szczurów rasy Zucker cechujących się hiperinsulinemią i insulinoopornością[15]. Na podstawie powyższych obserwacji można stwierdzić, że insulina uczestniczy w regulacji przepuszczalności kłębuszkowej bariery filtracyjnej, zaś zaburzenia w transdukcji sygnału insulinowego mogą prowadzić do zmian w funkcjonowaniu podocytów, zwiększenia przepuszczalności bariery filtracyjnej i rozwoju albuminurii. W związku z tym istotne jest poznanie szlaków sygnalizacyjnych zaangażowanych w regulację odpowiedzi komórek podocytarnych na działanie insuliny.

Jony Ca^{2+} są wewnątrzkomórkowymi wtórnymi przekaźnikami sygnału[17,18]. Zmiany stężenia wapnia w komórce stanowią dla niej sygnał do rozpoczęcia wielu procesów, m. in. reorganizacji szkieletu aktynowego czy zmian ekspresji genów[17]. Napływ jonów Ca^{2+} do komórki podocytarnej regulowany jest poprzez pompy oraz kanały wapniowe, wśród których znajdują się kanały jonowe TRPC6 należące do rodziny białek TRP.

Rodzina białek TRP stanowi grupę nieselektywnych kanałów kationowych, podzieloną na sześć podrodzin, w oparciu o homologię aminokwasową: TRP**C** (***C****anonical*), TRP**M** (***M****elastatin*), TRP**V** (***V****anilloid*), TRP**A** (***A****nkyrin*), TRP**P** (***P****olycystic*), TRP**ML** (***M****ucol****i****pin*)[19]. U ssaków, kanały z podrodziny kanonicznej TRP wykazują największą zgodność z kanałem TRP muszki owocowej[20]. W obrębie grupy kanałów TRPC wyróżnia się cztery podgrupy: 1) TRPC1, 2) TRPC2, 3) TRPC3/TRPC6/TRPC7 oraz 4) TRPC4/TRPC5[20]. Kanały TRPC zbudowane są z czterech podjednostek, z których każda składa się z sześciu domen transbłonowych. Pomiędzy piątą i szóstą domeną występuje pętla tworząca por, przez który przepływają jony[21]. Cechą charakterystyczną kanałów TRPC są cztery domeny ankirynowe zlokalizowane na N-końcu i krótka domena TRP na C-końcu[21–23]. Uważa się, że pobudzenie kanałów TRPC jest związane z aktywacją szlaku sygnałowego zależnego od fosfolipazy C, przy czym podrzędne szlaki przekazywania sygnału przez poszczególnych członków tej grupy mogą się różnić[24].

Białko TRPC6, należące do podrodziny kanałów regulowanych przez receptory (ROCs), jest nieselektywnym kanałem kationowym charakteryzującym się 6-krotnie większą przepuszczalnością dla jonów Ca^{2+} niż dla jonów Na^{+} [25].

W podocytach kanały TRPC6 zlokalizowane są głównie w SD, gdzie oddziałując z nefryną i synaptopodyną kontrolują organizację filamentów aktynowych i wielkość wypustek stopowatych[9,26,27]. Kanały TRPC6 znajdują się również w błonie plazmatycznej ciała komórki oraz wypustkach stopowatych podocytów, odgrywając istotną rolę w utrzymaniu komórkowej homeostazy wapnia[9].

Zmiany w ilości kanałów TRPC6 na powierzchni podocytów są związane z działaniem insuliny[28,29]. Pod wpływem insuliny w podocytach zwiększa się aktywność oksydazy NAD(P)H, która odpowiada za produkcję reaktywnych formy tlenu (ROS)[15,28]. Nagromadzenie ROS w wyniku insulinozależnej aktywacji oksydazy NAD(P)H prowadzi do zwiększenia ilości kanałów TRPC6 w błonie komórkowej podocytów[28,30]. Z kolei wzrost ekspresji białka lub aktywacja TRPC6 odpowiadają za nadmierny napływ jonów Ca^{2+} do podocytów[26], prowadząc do reorganizacji filamentów aktynowych i zaburzeń w funkcjonowaniu kłębuszkowej bariery filtracyjnej[29]. U szczurów chorych na cukrzycę typu 1 nokaut TRPC6 prowadzi do obniżenia $[Ca^{2+}]_i$ oraz ograniczenia uszkodzeń wypustek stopowatych podocytów w progresji DKD[32]. Ponadto nokautowi TRPC6 towarzyszy zmniejszenie ilości nefryny w moczu[32], będącej markerem uszkodzenia błony filtracyjnej kłębuszka[33].

W negatywną regulację aktywności kanału TRPC6 zaangażowany jest szlak sygnałowy NO/cGMP/PKG, gdzie PKG-zależna fosforylacja kanału TRPC6 w pozycji Thr69 hamuje napływ jonów Ca^{2+} do komórki[34,35]. W podocytach zaobserwowano występowanie podobnego mechanizmu regulacji aktywności kanału TRPC6, z którym wiąże się ograniczenie ruchliwości komórek[36].

Dotychczasowe badania wykazały istotną rolę kanału TRPC6 i jonów Ca^{2+} w dynamicznej reorganizacji filamentów aktynowych w podocytach. Badania te wymagają kontynuacji, ponieważ jak dotąd słabo poznany jest sposób, w jaki białka poszczególnych szlaków sygnałowych współdziałają z jonami Ca^{2+} w kontrolowaniu dynamiki filamentów aktynowych tych komórek oraz w regulacji przepuszczalności kłębuszkowej bariery filtracyjnej.

Prawidłowe funkcjonowanie podocytów zależy w dużej mierze od stanu energetycznego komórki. Kluczowym enzymem odpowiedzialnym za utrzymanie komórkowej równowagi energetycznej jest aktywowana AMP kinaza białkowa (AMPK),

zbudowana z trzech różnych podjednostek: katalitycznej α i regulatorowych β i γ[37,38]. Jej podjednostka katalityczna AMPKα występuje w dwóch izoformach: α1 oraz α2[38,39]. Do klasycznej aktywacji AMPK dochodzi w warunkach niedoboru energetycznego, gdy wzrasta wewnątrzkomórkowy stosunek AMP do ATP[38]. AMP, wiążąc się z podjednostką regulatorową AMPKγ, allosterycznie aktywuje enzym, a tym samym umożliwia, nadrzędnej wobec AMPK, kinazie LKB1 fosforylację podjednostki AMPKα w pozycji Thr172[38,40].

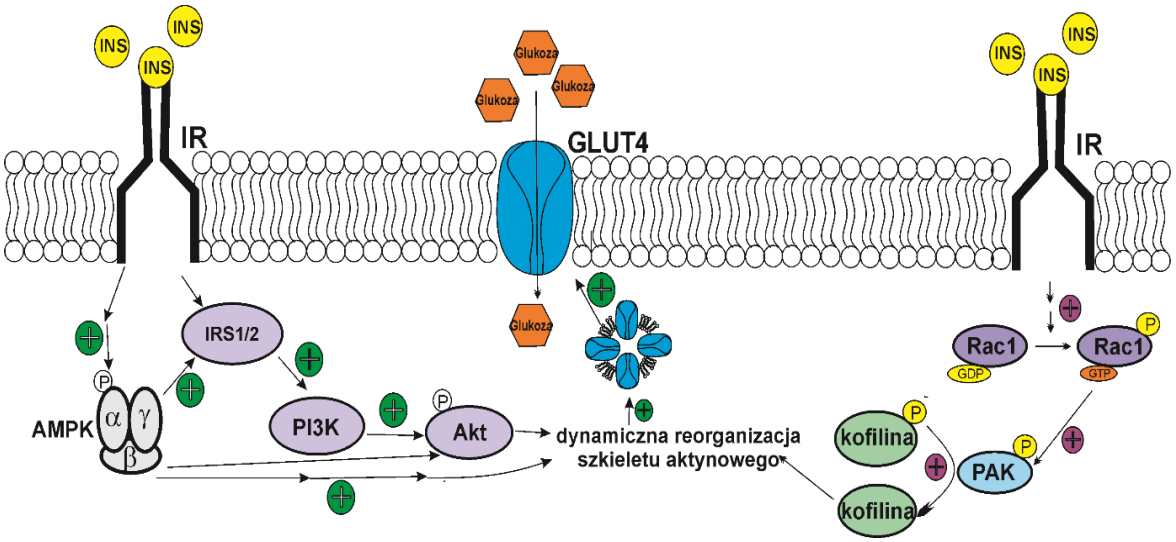
AMPK włącza się bezpośrednio w szlak sygnałowania insulinowego fosforylując w pozycji Ser789 białko IRS-1 będące substratem receptora insulinowego (IR)[41]. Dodatkowo AMPK aktywuje kinazę białkową B (Akt) promującą translokację transporterów glukozy GLUT4 do błony komórkowej[42,43] (Ryc. 1).

Badania naszego zespołu wykazały, że pod wpływem wysokiego stężenia glukozy w podocytach dochodzi do zahamowania aktywującego efektu insuliny wobec AMPK, któremu towarzyszy zniesienie stymulującego wpływu insuliny na dokomórkowy transport glukozy[44]. Dodatkowo badania przeprowadzone na kłębuszkach nerkowych izolowanych z nerek pacjentów chorych na cukrzycę wykazały zmniejszenie poziomu fosforylacji AMPK, sugerując że obniżona aktywność AMPK w cukrzycy może przyczyniać się do rozwoju DKD[45].

Alternatywnym szlakiem związanym z regulacją aktywności AMPK jest szlak sygnałowy zależny od jonów Ca^{2+}. Wzrost $[Ca^{2+}]_i$ prowadzi do aktywacji kinazy CaMKK-β, która fosforyluje AMPKα w pozycji Thr172 niezależnie od wewnątrzkomórkowego stosunku AMP do ATP[46]. Jak już wcześniej wspomniano, za regulację $[Ca^{2+}]_i$ odpowiadają kanały jonowe TRPC6[24,27]. Mimo to nie jest znany wpływ tego kanału na aktywność AMPK oraz jego rola w regulacji insulinozależnego dokomórkowego transportu glukozy w szczurzych podocytach. Dlatego też zbadanie roli szlaku sygnałowego TRPC6/AMPK w podocytach jest jednym z ważniejszych nowatorskich osiągnięć niniejszej pracy doktorskiej.

W regulacji dokomórkowego transportu glukozy bierze udział również białko Rac1, zaangażowane w procesy inicjujące przebudowę szkieletu aktynowego[47–49]. Wykazano, że w komórkach mięśni szkieletowych, pod wpływem insuliny, wzrasta zależna od Rac1 fosforylacja kinazy PAK, która również uczestniczy w reorganizacji filamentów aktynowych[48]. Ponadto pod wpływem insuliny stwierdzono także wzrost zależnej od kinazy PAK aktywności kofiliny, która jest białkiem bezpośrednio oddziałującym z aktyną[50]. Reorganizacja szkieletu aktynowego, polegająca na jego cyklicznej polimeryzacji

i depolimeryzacji, jest wymagana do efektywnego wprowadzenia pęcherzyków zawierających GLUT4 do błony komórkowej[51] (Ryc. 1). Zatem zmiany w funkcjonowaniu poszczególnych białek powyższego szlaku mogą prowadzić do zaburzeń translokacji transportera GLUT4. Jednym z kolejnych celów niniejszej dysertacji było zbadanie roli szlaku sygnałowego Rac1/PAK/kofilina w regulacji insulinozależnego dokomórkowego transportu glukozy w szczurzych podocytach.



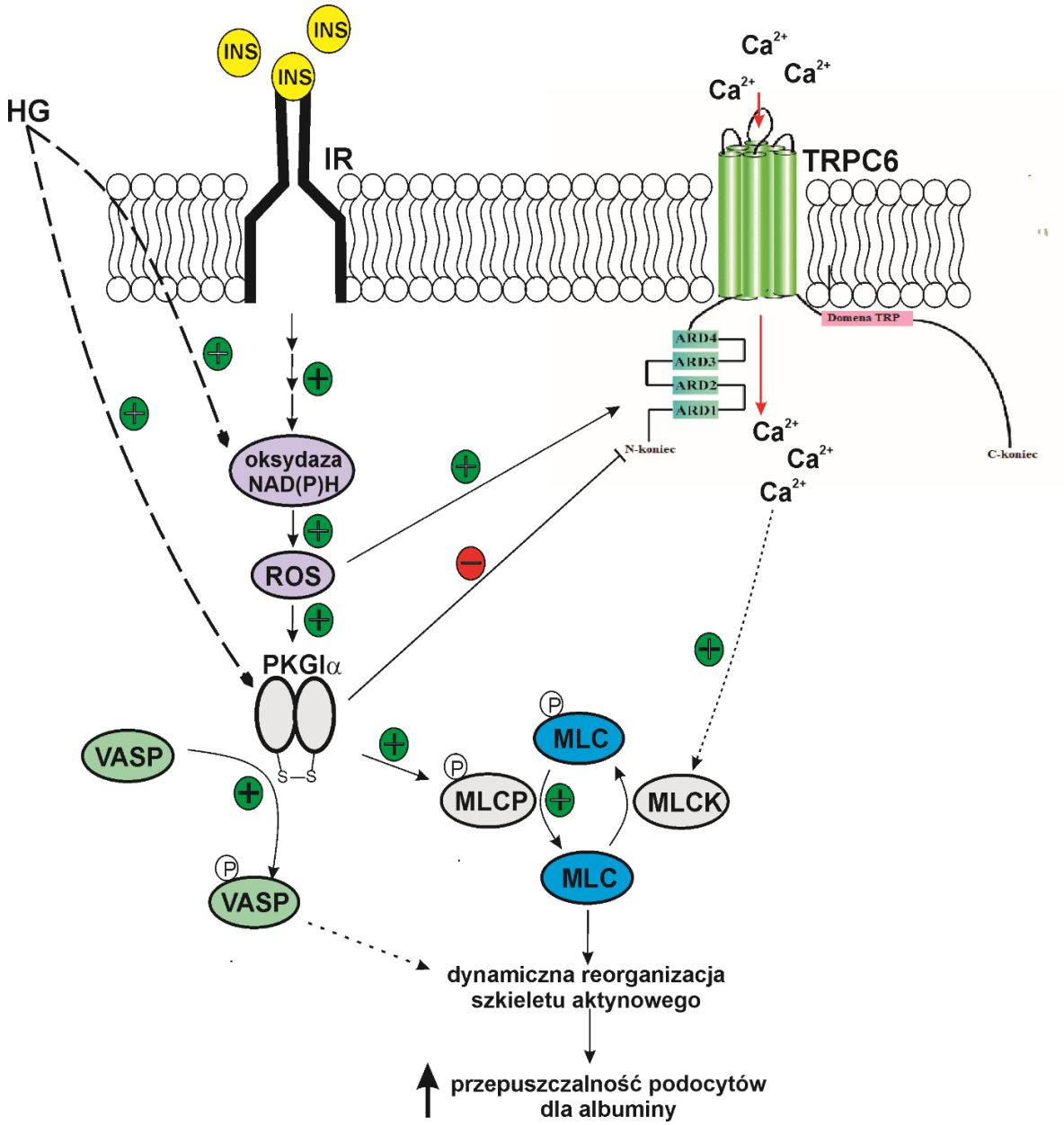
Ryc. 1. Udział AMPK oraz szlaku sygnałowego Rac1/PAK/kofilina w regulacji insulinozależnego dokomórkowego transportu glukozy.

Zależna od cGMP kinaza białkowa G typu Iα (PKGIα), pośrednicząc w przekazywaniu sygnału z IR do szkieletu aktynowego podocytów, odgrywa kluczową rolę w regulacji przepuszczalności bariery filtracyjnej[15,52]. PKGIα jest homodimerem zlokalizowanym w cytoplazmie komórki. Do aktywacji tego enzymu może dochodzić nie tylko w sposób klasyczny, zależny od cGMP, ale również niezależnie od cGMP wskutek dimeryzacji, polegającej na wytworzeniu mostków disiarczkowych pomiędzy sąsiadującymi resztami Cys42 monomerów PKGIα[53,54]. Badania przeprowadzone w naszym zespole wykazały, że w podocytach pod wpływem insuliny rośnie aktywność oksydazy NOX4 (izoforma oksydazy NAD(P)H), prowadząc do wzrostu ilości ROS, a w konsekwencji do dimeryzacji PKGIα[15,55,56]. Dodatkowo insulina reguluje aktywność kinazy łańcuchów lekkich miozyny (MLCK) poprzez PKGIα-zależną fosforylację docelowej podjednostki 1 fosfatazy miozyny (MYPT1) w pozycji Ser695 i Ser852, co zmniejsza stopień fosforylacji miozyny, a tym samym wpływa na aparat kurczliwy podocytów[15]. W istocie, zależne od PKGIα zmniejszenie poziomu fosforylacji łańcuchów

lekkich miozyny (MLC) oraz wzrost insulinozależnej fosforylacji MYPT1 przekłada się na zmiany w organizacji szkieletu aktynowego w komórkach podocytarnych oraz na zwiększoną przepuszczalność monowarstwy podocytów dla albuminy[15,55] (Ryc. 2). Podobne wyniki uzyskano dla podocytów eksponowanych na wysokie stężenia glukozy[16,57].

Otrzymane wyniki sugerują, że insulina oraz glukoza, aktywując szlak sygnałowy zależny od PKGIα, inicjują rozkurcz aparatu kurczliwego, co może przyczyniać się do zanikania wypustek stopowatych na skutek reorganizacji aktyny, a w konsekwencji do utraty prawidłowej funkcji podocytów i zwiększenia przepuszczalności bariery filtracyjnej. Powyższą hipotezę wydają się potwierdzać wyniki badań przeprowadzonych na zwierzęcym modelu charakteryzującym się insulinoopornością oraz hiperinsulinemią (szczury szczepu Zucker), w których występowanie albuminurii jest związane ze zwiększoną ilością białka PKGIα oraz oksydazy NOX4 w kłębuszkach nerkowych[15]. Również w badaniach funkcjonalnych, przeprowadzonych na izolowanych kłębuszkach nerkowych insulinoopornych zwierząt z hiperinsulinemią, wykazano zwiększoną przepuszczalność dla albuminy[15]. Liczne badania naszego zespołu potwierdzają również występowanie zależności pomiędzy aktywnością PKGIα, reorganizacją szkieletu aktynowego, a przepuszczalnością monowarstwy podocytów dla albuminy. Mechanizmy łączące aktywność PKGIα z białkami związanymi bezpośrednio z reorganizacją szkieletu aktynowego w wypustkach stopowatych podocytów pozostają nadal nieznane.

Białko VASP bierze udział w procesie wydłużania filamentów aktynowych i migracji komórki[58]. Fosforylacja białka VASP, która jest związana ze zmianą jego lokalizacji w obrębie komórki, 40-krotnie zmniejsza powinowactwo VASP do aktyny, wpływając na proces polimeryzacji filamentów[59–61]. W komórkach mięśni gładkich, PKG fosforylując VASP w pozycji Ser239, bierze udział w rozkurczu[62]. Niewiele wiadomo na temat ścieżek sygnałowych zależnych od białka VASP w podocytach. Morfologia, funkcja i zmiany w sieci aktynowej są w podocytach ściśle powiązane, a zaburzenia w organizacji aktyny mają bezpośrednie przełożenie na zanik wypustek stopowatych. Ostatnio wykazano, że wzrost fosforylacji białka VASP, w wyniku zahamowania fosfatazy PAR1, skutkuje zwiększoną ruchliwością podocytów[63]. Stwierdzono również, że w przypadku komórek śródbłonka, białko VASP jest odpowiedzialne za regulację ich przepuszczalności dla dekstranu[64], co pokazuje nową zależność pomiędzy fosforylacją białka VASP, regulacją organizacji filamentów aktynowych, a przepuszczalnością. Istotne było zatem zbadanie roli białka VASP w regulacji organizacji cytoszkieletu w podocytach i jego wpływu na przepuszczalność kłębuszkowej bariery filtracyjnej.



Ryc. 2. Wpływ insuliny na aktywność kanału TRPC6 oraz szlak sygnałowy zależny od PKGIα w komórkach podocytarnych.

4. Cel pracy

Celem pracy było określenie roli szlaku sygnałowego TRPC6/AMPK w insulinozależnej reorganizacji szkieletu aktynowego i w dokomórkowym transporcie glukozy w szczurzych podocytach, a także zbadanie udziału kanału jonowego TRPC6 i białka VASP w PKGIα-zależnej regulacji przepuszczalności kłębuszkowej bariery filtracyjnej w odpowiedzi na wysokie stężenia insuliny i/lub glukozy.

4.1. Cele szczegółowe

1. Zbadanie wpływu TRPC6 na insulinozależną:
   - regulację aktywności AMPK i PKGIα,
   - regulację dokomórkowego transportu glukozy,
   - aktywację białek szlaku sygnałowego Rac1/PAK/kofilina,
   - regulację przepuszczalności kłębuszkowej bariery filtracyjnej dla albuminy w wyniku aktywacji szlaku sygnałowego związanego z PKGIα.
2. Ocena wpływu insuliny na wzajemne oddziaływanie TRPC6 i AMPKα oraz TRPC6 i PKGIα w szczurzych podocytach.
3. Zbadanie roli TRPC6 oraz AMPK w insulinozależnej regulacji aktywności białka Rac1 i jego szlaku sygnałowego.
4. Określenie roli insuliny w PKGIα-zależnej fosforylacji białka VASP.
5. Wyjaśnienie wpływu wysokiego stężenia glukozy oraz insuliny na fosforylację białka VASP w pozycji Ser239 w szczurzych podocytach.
6. Zbadanie udziału białka VASP w regulacji dynamicznej reorganizacji szkieletu aktynowego oraz w przepuszczalności dla albuminy warstwy podocytów eksponowanych na wysokie stężenia insuliny oraz glukozy.

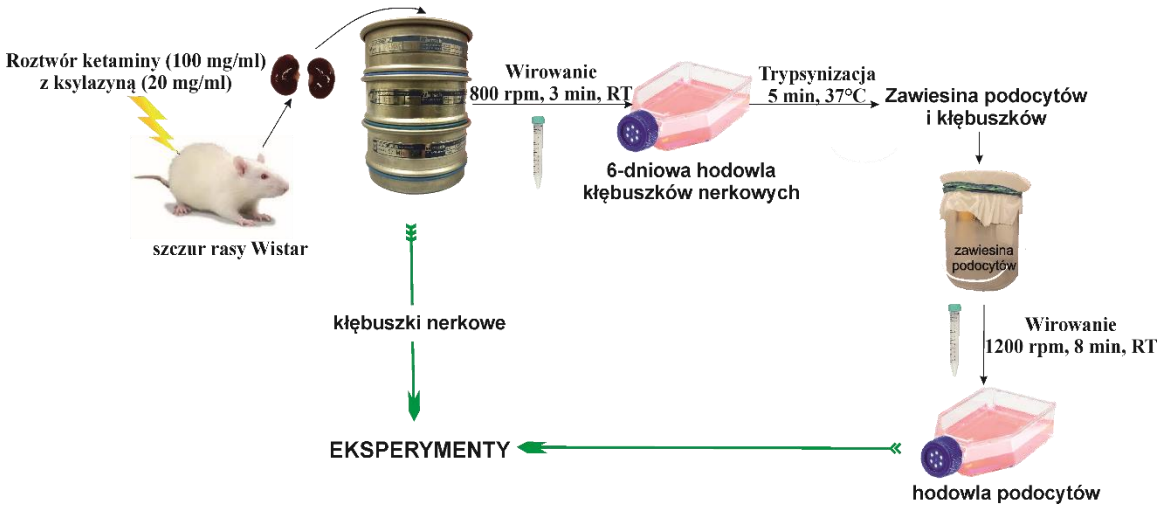
4.2. Uzasadnienie połączenia wskazanych prac w jeden zbiór

Insulina jest istotnym hormonem determinującym prawidłową morfologię oraz funkcjonowanie podocytów, dlatego też tematyka prac badawczych będących podstawą niniejszej rozprawy doktorskiej dotyczy roli insuliny w regulacji szlaków sygnałowych białek kluczowych dla prawidłowego funkcjonowania podocytów, a także przedstawia udział tego hormonu i wysokiego stężenia glukozy w patogenezie zaburzeń przepuszczalności kłębuszkowej bariery filtracyjnej i izolowanych podocytów.

5. Metodyka

Przygotowanie i hodowla szczurzych podocytów

Badania prowadzono na hodowli pierwotnej szczurzych podocytów oraz szczurzych kłębuszkach nerkowych otrzymywanych w oparciu o standardową procedurę opracowaną w Instytucie Anatomii i Biologii Komórki Uniwersytetu w Heidelbergu, Niemcy[65] (Ryc. 3).



Ryc. 3. Schemat otrzymywania kłębuszków nerkowych i hodowli pierwotnej szczurzych podocytów.

Doświadczenia prowadzono w układach badawczych, w których podocyty inkubowano w następujących warunkach:

- insulina (INS, 300 nM, 5 min), La^{3+} (1 mM, 15 min preinkubacja), Caloxin2A1 (0,3 mM, 15 min preinkubacja), CB-DMB (50 μM, 15 min preinkubacja), SKF96365 (inhibitor TRPC, 10 μM, 15 min preinkubacja), SAR7334 (specyficzny inhibitor TRPC6, 1 μM, 15 min), metformina (MTF, aktywator AMPK, 2 mM, 2 h), związek C (CC, inhibitor AMPK, 100 μM, 2 h);
- INS (300 nM, 5 min), OAG (1-oleilo-2-acetyloglicerol ,aktywator TRPC, 100 μM, 5 min), SKF96365 (10 μM, 15 min preinkubacja), 8-Br-cGMP (aktywator PKG, 100 μM, 5 min), Rp-8-Br-cGMPS (inhibitor PKG, 100 μM, 15 min preinkubacja), nadtlenek wodoru (H_2O_2, 100 μM, 5 min);
- standardowego (SG, 11 mM) lub wysokiego (HG, 30 mM) stężenia glukozy przez okres 5 dni w obecności odpowiednich modulatorów aktywności PKGIα (INS, 300 nM, 5 min lub 5 dni; 8-Br-cGMP, 100 μM, 5 min; Rp-8-Br-cGMPS, 100 μM, 5 dni).

Wcześniejsze wyniki badań wskazują, że 5-dniowa ekspozycja podocytów na HG prowadzi do rozwoju insulinooporności, manifestującej się zniesieniem stymulującego wpływu insuliny na dokomórkowy transport glukozy[14].

Przeprowadzono również doświadczenia w układach badawczych, w których izolowane kłębuszki nerkowe inkubowano w poniższych warunkach: INS (300 nM, 5 min), OAG (100 μM, 5 min) oraz SKF96365 (10 μM, 15 min preinkubacja).

Western Blot

W celu detekcji badanych białek lizat (15-20 μg białka na ścieżkę) nanoszono na 10% żel poliakrylamidowy i rozdzielano przy użyciu elektroforezy SDS-PAGE. Rozdzielone białka według masy cząsteczkowej przenoszono na nitrocelulozowe membrany za pomocą elektrotransferu. W celu potwierdzenia obecności badanych białek stosowano specyficzne przeciwciała I-rzędowe: anty-p-IRβ (Tyr1150-1151) (1:200, Santa Cruz Biotechnology), anty-IRβ (1:200, Santa Cruz Biotechnology), anty-p-Akt1/2/3 (Ser473) (1:400, Santa Cruz Biotechnology), anty-Akt1/2/3 (1:400, Santa Cruz Biotechnology), anty-p-MLC2 (Ser19) (1:400, Cell Signaling Technology), anty-MLC2 (1:1000, Cell Signaling Technology), anty-PKGIα (1:400, Santa Cruz Biotechnology), anty-p-MYPT1 (Ser965) (1:400, Santa Cruz Biotechnology), anty-MYPT1 (1:400, Santa Cruz Biotechnology), anty-TRPC6 (1:1000, Sigma-Aldrich), anty-p-PAK1/2/3 (Thr423/402/421) (1:800, Sigma-Aldrich), anty-p-PAK1/2/3 (Ser144/141/139) (1:800, Sigma-Aldrich), anty-PAK1/2/3 (1:800, Cell Signaling Technology), anty-p-kofilina (Ser3) (1:1000, Sigma-Aldrich), anty-kofilina (1:1000, Santa Cruz Biotechnology), anty-p-Rac1 (Ser71) (1:1000, OriGene), anty-Rac1 (1:875, Invitrogen), anty-ROCK1 (1:1000, Cell Siganling Technology), anty-ROCK2 (1:1000, Cell Siganling Technology), anty-p-AMPK (Thr172) (1:1000, Cell Signaling Technology), anty-p-AMPKα1 (1:500, Santa Cruz Biotechnology), anty-p-AMPKα2 (1:500, Santa Cruz Biotechnology), anty-AMPK (1:1000, Cell Signaling Technology), anty-pVASP (Ser239) (1:667, Abcam), anty-VASP (1:28 000, Sigma-Aldrich), anty-aktyna (1:16000, Sigma Aldrich).

Immunofluorescencja

Aby określić kolokalizację badanych białek, podocyty utrwalano 4% paraformaldehydem i płukano ogrzanym do 37ºC PBS. Następnie komórki kolejno permeabilizowano 0,1% Triton-X, płukano PBS, blokowano (PBS z 2% BSA, 2% FBS oraz 0,2% rybią żelatyną) i inkubowano ze specyficznymi przeciwciałami I-rzędowymi: anty-TRPC6 (1:100),

anty-PKGIα (1:15), anty-AMPKα1(1:100), anty-AMPKα2 (1:100), anty-kofilina (1:50), anty-VASP (1:30). Następnie przeciwciała I-rzędowe inkubowano z odpowiednimi przeciwciałami II-rzędowymi znakowanymi barwnikami fluorescencyjnymi. Zdjęcia podocytów wykonano za pomocą mikroskopu konfokalnego (Leica SP8X) z obiektywem imersyjnym 63x.

F-aktynę znakowano fluorescencyjnie falloidyną (1:200, Invitrogen). Zdjęcia podocytów z wybarwioną siecią aktynową wykonano za pomocą mikroskopu fluorescencyjnego (Nikon Ti Eclipse) z obiektywem 40x.

Transfekcja komórek siRNA

Wyciszanie ekspresji genu kodującego kanał wapniowy TRPC6, AMPKα1, AMPKα2, VASP, NOX4, NOX2 przeprowadzano za pomocą specyficznych siRNA. Wyniki interpretowano w odniesieniu do kontrolnego siRNA (scrambled siRNA, kontrola negatywna). Poziom wyciszenia określonego genu sprawdzano przeprowadzając analizę ilości odpowiedniego białka metodą Western Blot.

Izolacja RNA i real-time PCR

Podocyty homogenizowano i izolowano RNA z użyciem odczynnika TRI Reagent (Sigma). Całkowite RNA ekstrahowano metodą fenolowo-chloroformową. Ilość oraz jakość wyizolowanego RNA oznaczano spektrofotometrycznie przy długości fali 260 nm oraz 280 nm. Poziom badanego transkryptu analizowano metodą PCR w czasie rzeczywistym z wykorzystaniem komercyjnego zestawu LightCycler DNA SYBR Green I, za pomocą termocyklera LightCycler 480 (Roche Diagnostics).

Wychwyt glukozy

Podocyty inkubowano w obecności niespecyficznego inhibitora TRPC (SKF96365, 100 μM, 15 min) lub specyficznego inhibitora TRPC6 (SAR7334, 1 μM, 15 min). Następnie do komórek dodawano 1 μCi [1,2-^{3}H]-deoksy-D-glukozy rozcieńczonej w nieradioaktywnym roztworze glukozy (stężenie końcowe 50 μM) z dodatkiem lub bez 300 nM insuliny (3 min w 37°C, 5% CO2). Następnie płytkę umieszczano na lodzie i z każdego dołka pobierano mieszaninę inkubacyjną do naczynek scyntylacyjnych zawierających płyn scyntylacyjny w celu oznaczenia radioaktywności zewnętrznej. Podocyty przepłukiwano schłodzonym do 4^{o}C PBS, a następnie traktowano 0,05 N NaOH w celu ich lizy. Uzyskane lizaty komórkowe przenoszono do naczynek scyntylacyjnych z płynem scyntylacyjnym w celu oznaczenia

radioaktywności wewnątrzkomórkowej. Radioaktywność mierzono za pomocą licznika scyntylacyjnego (MicroBeta2®, Perkin Elmer, Waltham, USA).

Immunoprecypitacja

W celu wyodrębnienia badanego białka z mieszaniny innych białek, lizaty komórkowe inkubowano z komercyjnie dostępnym odczynnikiem Protein A/G-PLUS Agarose z odpowiednimi przeciwciałami I-rzędowymi. Otrzymaną próbkę analizowano przy pomocy metody Western blot.

Przepuszczalność kłębuszków nerkowych dla albuminy

Kłębuszki nerkowe inkubowano w pożywce z 5% BSA w obecności lub bez insuliny, OAG, SKF96365. Kłębuszki kontrolne inkubowano z dodatkiem buforu zawierającego 5% BSA. Zmiany w objętości kłębuszków rejestrowano za pomocą mikroskopu (Olympus IX51) 1 min przed i po dodaniu badanego związku. Na podstawie otrzymanych wyników wyliczano konwekcyjny współczynnik przepuszczalność dla albuminy ($P_{alb}=1-\sigma_{alb}$, gdzie σ_{alb} - współczynnik przepuszczalności) określający ruch albuminy jako konsekwencję przemieszczania się wody w gradiencie onkotycznym oraz przepuszczalność kapilar kłębuszka nerkowego dla albuminy.

Oznacznie G-LISA

Do oznaczenia aktywności Rac1 użyto komercyjnie dostępnego zestawu G-LISA Rac1 Activation Biochem Kit –Absorbance Based (Cytoskeleton Inc, Denver, USA).

Pomiar stężenia cGMP

Do oznaczenia stężenia cGMP w lizatach podocytów użyto komercyjnie dostępnego zestawu Cyclic GMP EIA Kit (Cayman Chemical Company, Michigan, USA).

Oznaczanie aktywności oksydazy NAD(P)H

Aktywność oksydazy NAD(P)H mierzono zmodyfikowaną metodą chemiluminescencyjną zależną od lucygeniny. W celu zbadania produkcji anionu nadtlenku, do wzbogaconego o EDTA i lucygeninę buforu PBS dodawano homogenat komórkowy (50 μg białka). Reakcję rozpoczynano przez dodanie NADPH. Emisję fotonów, w przeliczeniu na relatywną jednostkę światła, mierzono za pomocą luminometru FB12 (Berthold).

Analizy statystyczne

Wyniki przedstawiają średnią ± standardowy błąd średniej (SEM). Obliczenia statystyczne wykonywano stosując analizę wariancji (ANOVA) testem t-Studenta-Newmana-Keulsa lub testem t-studenta dla grup niezależnych wykorzystując oprogramowanie SigmaPlot 11.0 (Systat Software, Inc., USA). Istotność statystyczną ustalono na poziomie $p<0,05$.

6. Omówienie i podsumowanie najważniejszych wyników

6.1. Rola TRPC6 w regulacji przepuszczalności kłębuszkowej bariery filtracyjnej na drodze insulinozależnej aktywacji szlaku sygnałowego związanego z PKGIα

Publikacja 1[66]

Kłębuszkowa bariera filtracyjna odgrywa istotną rolę w funkcji filtracyjnej nerek. Jednym z istotnych czynników regulujących przepuszczalność kłębuszkowej bariery filtracyjnej jest insulina – hormon wpływający na funkcję oraz morfologię podocytów. Wyniki badań dowodzą, że insulina, poprzez aktywację PKGIα, zwiększa przepuszczalność dla albuminy zarówno kłębuszkowej bariery filtracyjnej, jak i monowarstwy podocytów utworzonej przez komórki hodowane na nylonowych membranach pokrytych kolagenem typu IV[15]. PKGIα-zależna regulacja przepuszczalności monowarstwy podocytów dla albuminy jest skorelowana ze zmianami $[Ca^{2+}]_i$[67]. Badania przeprowadzone przez *Möller i wsp. (2007)* wykazały, że nadekspresja genu kodującego kanał TRPC6 u myszy prowadzi do zwiększenia ilość kanałów TRPC6 w wypustkach stopowatych podocytów w bliskim sąsiedztwie SD, z towarzyszącą proteinurią[68]. Kanał TRPC6 jest odpowiedzialny za napływ jonów Ca^{2+} do komórki podocytarnej, a zatem może wpływać na przepuszczalność kłębuszkowej bariery filtracyjnej.

W związku z powyższym sprawdzono, czy insulina reguluje przepuszczalność bariery filtracyjnej w wyniku TRPC6-zależnej aktywacji szlaku sygnałowego związanego z PKGIα. Nasze wcześniejsze prace dowodzą, że insulina zwiększa przepuszczalność kłębuszkowej bariery filtracyjnej[15,16]. Badania przeprowadzone w ramach niniejszej dysertacji wykazały, że inkubacja kłębuszków nerkowych z insuliną w obecności inhibitora kanału TRPC (SKF96365) prowadzi do zahamowania działania insuliny i zmniejszenia przepuszczalności kłębuszkowej bariery filtracyjnej (Fig. 1A), podczas gdy zastosowanie aktywatora kanału TRPC (OAG) zwiększa przepuszczalność bariery filtracyjnej dla albuminy (Fig. 1B). Podobne wyniki uzyskano w badaniach, w których stosowano komórki podocytarne. Pod wpływem inhibitora SKF96365 obserwowano zniesienie stymulującego wpływu insuliny na przepuszczalność monowarstwy podocytów dla albuminy, natomiast zastosowanie aktywatora kanału TRPC powodowało zwiększenie przepuszczalności (Fig. 1C-D). Zaobserwowano również, że zastosowanie aktywatora PKG (8-Br-cGMP) zwiększa przepuszczalność monowarstwy podocytów dla albuminy. Wyciszenie genu kodującego TRPC6 za pomocą wyciszającego RNA (siRNA) znosi działanie insuliny oraz 8-Br-cGMP

na przepuszczalność monowarstwy podocytów dla albuminy (Fig. 10A). Wyciszenie ekspresji genu kodującego PKGIα za pomocą siRNA również prowadziło do zniesienia działania insuliny oraz zmniejszenia wpływu aktywatora kanału TRPC (OAG) na przepływ albuminy przez monowarstwę podocytów (Fig. 10B).

Kim i wsp. (2012) wykazali, że insulina zwiększa ilość kanałów TRPC6 i podjednostki NOX4 w błonie podocytów. Wyciszenie ekspresji genu kodującego NOX4 w mysich podocytach znosiło stymulujący wpływ insuliny na kanał TRPC6[28]. Wyniki badań przeprowadzonych przez *Ilatovskaya i wsp. (2018)* pokazały, że NOX4-zależna produkcja ROS prowadzi do uszkodzenia podocytów w wyniku zwiększonego napływu jonów Ca^{2+} do komórki przez kanały TRPC6[30]. Inkubacja podocytów z wysokim stężeniem glukozy[69], aminonukleozydem puromycyny[70] lub angiotensyną II[71] również prowadzi do wzrostu produkcji ROS i zwiększenia TRPC6-zależnego napływ jonów Ca^{2+} do komórek. Przedstawione powyżej wyniki badań sugerują, że wpływ insuliny na aktywność kanału TRPC6 zależy od produkcji ROS w wyniku aktywacji oksydazy NOX4.

W związku z tym zbadano wpływ insuliny, OAG oraz SKF96365 na aktywność oksydazy NAD(P)H w szczurzych podocytach. Zarówno insulina, jak i OAG zwiększają aktywność tego enzymu. Preinkubacja podocytów z inhibitorem TRPC zmniejsza aktywność oksydazy NAD(P)H o 55% oraz znosi stymulujący wpływ insuliny na aktywność tego enzymu (Fig. 2A). Ponadto podocyty z wyciszoną ekspresją genu kodującego NOX4 charakteryzują się znacząco zmniejszoną aktywnością oksydazy NAD(P)H, mimo obecności OAG (Fig. 2B). Specyficzne wyciszenie ekspresji genu kodującego TRPC6 skutkuje zniesieniem stymulującego wpływu insuliny na aktywność oksydazy NAD(P)H (Fig. 2C, D). Dodatkowo zaobserwowano, że insulina zwiększa $[Ca^{2+}]_i$ o 60%, a inhibitor TRPC SKF96365 hamuje ten efekt (Fig. 2E, F).

Nasze poprzednie wyniki badań wykazały, że aktywacja oksydazy NAD(P)H w odpowiedzi na insulinę jest skorelowana ze wzrostem aktywności PKGIα w podocytach[15]. Dlatego też zbadano, czy kanały jonowe TRPC regulują aktywność PKGIα. Eksperymenty przeprowadzone w ramach niniejszej pracy doktorskiej dowodzą, że insulina powoduje wzrost dimeryzacji PKGIα zależnie od kanału TRPC (Fig. 3).

Szlak sygnałowy zależny od PKGIα, a także TRPC6-zależny napływ jonów Ca^{2+} wpływają na aktywność aparatu kurczliwego podocytów, regulując zmiany w organizacji szkieletu aktynowego[55,68]. W związku z powyższym założono, że aktywacja kanału TRPC powoduje zmiany w poziomie fosforylacji MYPT1 i MLC w wyniku aktywacji PKGIα. Wykazano, że zastosowanie inhibitora TRPC znosi efekt insuliny na stopień fosforylacji

białek MYPT1 oraz MLC (Fig. 4A, B), natomiast OAG-zależna aktywacja kanału TRPC zwiększa poziom fosforylacji białka MYPT1 o 58% oraz prowadzi do defosforylacji białka MLC (Fig. 4C, D). Stwierdzono również, że w obecności zarówno insuliny, jak i aktywatora TRPC dochodzi do reorganizacji szkieletu aktynowego podocytów i zaniku włókien naprężeniowych. Podocyty inkubowane z insuliną w obecności inhibitora TRPC charakteryzują się widocznymi włóknami naprężeniowymi rozmieszonymi równomiernie w całej komórce (Fig. 5). Również nadekspresja genu kodującego białko TRPC6 skutkuje zanikiem włókien naprężeniowych w mysich podocytach[68].

Kolejnym etapem badań było określenie, czy TRPC6 wchodzi w interakcję z PKGIα. W naszym zespole po raz pierwszy udowodniono występowanie oddziaływań między TRPC6 i PKGIα. Co więcej, wykazano, że pod wpływem insuliny o 47% wzrasta ilość koimmunoprecypitatu TRPC6-PKGIα (Fig. 6). Dodatkowo barwienie immunofluorescencyjne podocytów eksponowanych na insulinę potwierdziło wzrost kolokalizacji TRPC6 i PKGIα o 24% (Fig. 7). Podobny efekt uzyskano stosując aktywator PKG (8-Br-cGMP) oraz H_2O_2. Wcześniejsze wyniki badań wykazały, że PKGIα wchodzi w interakcję z podjednostką Slo1 kanału potasowego aktywowanego przez jony wapnia o dużym przewodnictwie (BK_{Ca}), a insulina zwiększa ilość koimmunoprecypitatu PKGIα-Slo1[72]. Z kolei kanał BK_{Ca} tworzy koimmunoprecypitat z kanałem TRPC6 i innymi białkami SD[24]. Również barwienie immunofluorescencyjne mysich podocytów potwierdziło występowanie kolokalizacji pomiędzy podjednostką Slo1 i TRPC6[73]. *Kim i wsp. (2009)* sugerują, że TRPC6-zależny napływ jonów Ca^{2+} warunkuje aktywację kanału BK_{Ca}. W kontekście przedstawionych wyników badań można przypuszczać, że interakcje zachodzące pomiędzy PKGIα, TRPC6 i BK_{Ca} w podocytach są częścią większego kompleksu, który jest elementem mechanizmu stabilizującego filtrację kłębuszkową w obliczu różnych bodźców, np. insuliny.

Kolejnym krokiem było zbadanie, czy istnieje zależność pomiędzy ekspresją genu TRPC6 i insulinozależną regulacją aktywności PKGIα. Inkubacja podocytów z insuliną prowadzi do znaczącego spadku ekspresji TRPC6 na poziomie mRNA. Efekt ten był zahamowany w obecności inhibitora PKG (Rp-8-Br-cGMPS). Z kolei zastosowanie insuliny lub aktywatorów PKG (8-Br-cGMP, H_2O_2) zmniejszało ilość białka TRPC6 w podocytach (Fig. 8). Jak już wcześniej wspomniano, szlak sygnałowy NO/cGMP/PKG, poprzez fosforylację TRPC6 w pozycji Thr69, zmniejsza aktywność tego kanału. Również farmakologiczni agoniści PKG indukują fosforylację TRPC6 w pozycji Thr69[36]. Otrzymane

wyniki mogą wskazywać na istnienie mechanizmu adaptacyjnego, który przeciwdziała gromadzeniu się wapnia wewnątrz komórki i zmniejsza skurcz podocytów.

W ramach niniejszej pracy doktorskiej zbadano również rolę kanału TRPC6 w insulinozależnej aktywacji szlaku sygnałowego zależnego od PKGIα. Stwierdzono, że wyciszenie genu kodującego TRPC6 za pomocą siRNA zmniejsza ilość białka PKGIα w podocytach oraz uniemożliwia tworzenie wiązań disiarczkowych pomiędzy monomerami PKGIα pomimo obecności insuliny (Fig. 9A, B). Aktywacja PKGIα, w wyniku zastosowania 8-Br-cGMP lub insuliny, zwiększa fosforylację MYPT1 i redukuje ilość ufosforylowanej formy MLC. Wyciszenie ekspresji genu kodującego TRPC6 uniemożliwia insulinie oddziaływanie na powyższe białka. Poza tym wyciszenie ekspresji TRPC6 prowadzi do zmniejszenia stopnia fosforylacji MYPT1 oraz wzrostu stopnia fosforylacji MLC w obecności 8-Br-cGMP (Fig. 9C, D).

6.2. Określenie roli szlaku TRPC6/AMPK w insulinozależnej reorganizacji szkieletu aktynowego i dokomórkowym transporcie glukozy w szczurzych podocytach

Publikacja 2[74]

W pierwszym etapie badań zaplanowano określenie roli sygnalizacji wapniowej w regulacji transdukcji sygnału insulinowego w podocytach. Otrzymane wyniki badań wykazały, że insulina zwiększa stopień fosforylacji IR oraz Akt. Zahamowanie wypływu wapnia z komórki (po zastosowaniu La^{3+}, caloxin2A1, CB-DMB) nie ma wpływu na poziom fosforylacji IR ani Akt. Również inkubacja podocytów z powyższymi inhibitorami w obecności insuliny nie wpływa na poziom fosforylacji tych białek (Fig. 1).

Następnie zbadano wpływ inhibitora TRPC na transdukcję sygnału insulinowego. Podocyty poddane działaniu inhibitora SKF96365 charakteryzują się wprawdzie niezmienionym stopniem fosforylacji IR, jednakże stymulujący wpływ insuliny na stopień fosforylacji Akt jest zniesiony (Fig. 1).

W celu zbadania roli kanału TRPC6 w regulacji białek będących częścią szlaku sygnałowego zależnego od insuliny zastosowano specyficzne siRNA. Stwierdzono, że transfekcja podocytów za pomocą siRNA TRPC6 znacząco zmniejsza ilość białka TRPC6 (Fig. 2A) oraz hamuje stymulujące działanie insuliny na stopień fosforylacji Akt (Fig. 2B), bez zmian w fosforylacji IR (Fig. 2C).

W regulację szlaku sygnałowego zależnego od insuliny i stymulację insulinozależnego dokomórkowego transportu glukozy w podocytach zaangażowana jest również AMPK, która może być aktywowana pośrednio przez jony Ca^{2+} [39]. Uzyskane przez nas wyniki badań potwierdziły, że insulina indukuje wzrost stopnia fosforylacji AMPKα (Fig. 3A). Ponadto insulina indukuje napływ wapnia do komórek[28,66].W związku z tym postawiono hipotezę badawczą, że insulina wpływa na stopień fosforylacji AMPKα poprzez aktywację kanału TRPC. Zarówno zastosowanie inhibitora TRPC, jak i wyciszenie ekspresji genu kodującego TRPC6 znosiło efekt działania insuliny na stopień fosforylacji AMPK (Fig. 3A, B). Co więcej, wykorzystując metodę Western blot oraz transfekcję siRNA TRPC6 udowodniono, że kanał TRPC6 odgrywa znaczącą rolę w regulacji insulinozależnego transportu glukozy w podocytach (Fig. 3C-E). Udział kanałów TRPC w regulacji insulinozależnego dokomórkowego transportu glukozy potwierdził *Lanner i wsp. (2009)*. Wyciszenie ekspresji genu kodującego TRPC3 prowadzi do zahamowania tego procesu w komórkach mięśni szkieletowych[75]. Ponadto koimmunoprecypitacja białek wykazała,

że TRPC3 oddziałuje z GLUT4 we włóknach mięśniowych[75]. Otrzymane wyniki sugerują, że TRPC6-zależny napływ jonów Ca^{2+} może stymulować przemieszczanie się pęcherzyków zawierających GLUT4 do błony komórkowej podocytów, a w konsekwencji transport glukozy do komórki.

Kanał TRPC6 jest częścią kompleksu sygnałowego zlokalizowanego w SD[9]. Kompleks ten odgrywa istotną rolę w regulacji funkcji podocytów. W związku z tym postanowiono sprawdzić, czy w podocytach występuje interakcja pomiędzy TRPC6 i AMPKα. Przeprowadzając koimmunoprecypitację białek wykazano, że TRPC6 oddziałuje z obiema izoformami AMPKα (α1 oraz α2). Pod wpływem insuliny ilość koimmunoprecypitatu TRPC6-AMPKα2 wzrasta aż o 45%, podczas gdy ilość koimmunoprecypitatu TRPC6-AMPKα1 pozostaje bez zmian (Fig. 4A-C). Również barwienie immunofluorescencyjne podocytów eksponowanych na insulinę wykazuje zwiększenie kolokalizacji TRPC6 z AMPKα2 (Fig. 4D). *Bair i wsp. (2009)* wykazali także istnienie zależności pomiędzy AMPKα i TRPC, gdzie wyciszenie ekspresji genu kodującego TRPC1 znosi stymulujący wpływ peptydu agonisty PAR1 na fosforylację AMPKα w komórkach endotelialnych[76].

Insulinozależna aktywacja białka Rac1 indukuje reorganizację aktyny w mięśniach szkieletowych, czemu towarzyszy zwiększenie dokomórkowego transportu glukozy[48]. W związku z tym przyjęto założenie, że ograniczenie insulinozależnego transportu glukozy, w wyniku zahamowania aktywności kanałów TRPC6, może być skorelowane z ograniczeniem funkcji regulatorowej białka Rac1 w odniesieniu do szkieletu aktynowego podocytów. W naszych badaniach wykazano, że insulina aktywuje białko Rac1, natomiast inkubacja podocytów w obecności inhibitora TRPC znosi stymulujący wpływ insuliny na aktywność Rac1 (Fig. 5A).

Następnie, aby określić wpływ AMPK na białko Rac1 zastosowano aktywator AMPK (metformina, MTF) oraz inhibitor AMPK (związek C, CC). Użycie modulatorów aktywności AMPK potwierdza istotne znaczenie tego białka w regulacji transportu glukozy w podocytach (Fig. 5C). Co więcej, wykazano, że MTF istotnie statystycznie zwiększa zarówno aktywność, jak i stopień fosforylacji białka Rac1 (Fig. 5D, E). Aby sprawdzić która izoforma AMPKα jest zaangażowana w regulację aktywności Rac1, podocyty transfekowano siRNA AMPKα1 lub AMPKα2 (Fig. 6A-B). Wyniki badań potwierdziły, że tylko wyciszenie ekspresji AMPKα2 w podocytach traktowanych MTF skutkuje zmniejszeniem o 25% stopnia fosforylacji białka Rac1 (Fig. 6D).

Jednym z efektorów białka Rac1 związanym z regulacją organizacji aktyny jest kinaza PAK. Do aktywacji kinazy PAK dochodzi wskutek jej fosforylacji w pozycji Thr423 oraz Ser141, odłączenia dimeru i odsłonięcia domeny katalitycznej[77]. W podocytach, insulina zwiększa poziom fosforylacji kinazy PAK w pozycji Thr423 o 33% i Ser141 o 71% (Fig. 7A, C). Zarówno zastosowanie inhibitora SKF96365, jak i wyciszenie ekspresji TRPC6 znosi pozytywny wpływ insuliny na stopień fosforylacji kinazy PAK (Fig. 7A-D). Dodatkowo zbadano wpływ sygnalizacji wapniowej na poziom kinazy ROCK (izoformy ROCK1 i ROCK2), związanej z reorganizacją szkieletu aktynowego. W obecności inhibitora TRPC ilość białka ROCK1 i ROCK2 nie zmienia się (Fig. 8A, C). Natomiast transfekcja podocytów siRNA TRPC6 skutkuje zmniejszeniem ilości białka ROCK1 o 23%, bez zmian w ilości białka ROCK2 (Fig. 8B, D).

TRPC6-zależna regulacja $[Ca^{2+}]_i$ w podocytach odgrywa kluczową rolę w utrzymaniu prawidłowej struktury szkieletu aktynowego[31]. W związku z powyższym kolejnym etapem badań było sprawdzenie, czy kanały TRPC6 pośredniczą w procesie insulinozależnej regulacji dynamiki szkieletu aktynowego w podocytach. Badania przeprowadzone w ramach niniejszej pracy doktorskiej udowodniły, że insulina indukuje aktywację kofiliny w wyniku jej defosforylacji (Fig. 9A). Natomiast preinkubacja podocytów z inhibitorem TRPC - SKF96365 (Fig. 9A) lub transfekcja podocytów siRNA TRPC6 (Fig. 9B) znoszą ten efekt. Na podstawie analizy immunofluorescencyjnej kofiliny oraz aktyny w komórkach eksponowanych na insulinę, stwierdzono zwiększenie kolokalizacji kofiliny z aktyną o 20%. Preinkubacja podocytów z SKF96365 w obecności insuliny znosi ten efekt (Fig. 9C). Również w komórkach neuronalnych, aktywacja kanału TRPC wpływa pozytywnie na aktywność kofiliny[78].

6.3. Określenie roli szlaku sygnałowego PKGIα/VASP w zależnej od insuliny oraz wysokiego stężenia glukozy regulacji przepuszczalności monowarstwy podocytów dla albuminy

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Insulina oraz wysokie stężenie glukozy, aktywując zależny od PKGIα szlak sygnałowy, zwiększają przepuszczalność monowarstwy podocytów dla albuminy. W następnym etapie badań zbadano wpływ insuliny oraz aktywności PKG na ilość białka VASP i stopień jego fosforylacji w szczurzych podocytach. Metodą immunodetekcji stwierdzono, że zarówno pod wpływem insuliny, jak i farmakologicznej aktywacji PKG z zastosowaniem 8-Br-cGMP, wzrasta stopień fosforylacji białka VASP w pozycji Ser239, bez istotnych zmian w jego ilości (Fig. 1), podczas gdy zastosowanie inhibitora PKG (Rp-8-Br-cGMPS) istotnie zmniejsza stopień fosforylacji białka VASP (Fig. 1B). Wyniki naszych badań wykazały, że aktywator PKG, podobnie jak insulina, zwiększa kolokalizację PKGIα i białka VASP oraz wpływa na rozmieszczenie tych białek w komórce (Fig. 2).

Następnie zbadano, czy długotrwała inkubacja podocytów w obecności insuliny lub wysokiego stężenia glukozy wpływa na ilość białka VASP w komórce. Analiza Western blot wykazała, że insulina lub wysokie stężenie glukozy zwiększają ilość białka VASP odpowiednio o 35% i 29% (Fig. 3A). Ponadto zastosowanie powyższych warunków skutkuje zwiększeniem kolokalizacji białka VASP i PKGIα w podocytach (Fig. 3B). Dodatkowo podocyty inkubowano z inhibitorem PKG, aby potwierdzić istnienie zależności pomiędzy PKGIα i białkiem VASP w warunkach standardowego lub wysokiego stężenia glukozy. Metoda immunodetekcji wykazała, że zastosowanie inhibitora PKG zmniejsza ilość białka VASP w podocytach eksponowanych zarówno na standardowe, jak i wysokie stężenie glukozy (Fig. 4).

Następnie, aby potwierdzić PKGIα-zależną fosforylację białka VASP w pozycji Ser239, podocyty, eksponowane na standardowe lub wysokie stężenia glukozy, transfekowano siRNA PKGIα. W odpowiedzi na wysokie stężenie glukozy zaobserwowano wzrost stopnia fosforylacji białka VASP Ser239. Po wyciszeniu ekspresji genu PKGIα lub zastosowaniu inhibitora PKG wykazano istotny spadek stopnia fosforylacji białka VASP na Ser239 zarówno w warunkach standardowego, jak i wysokiego stężenia glukozy (Fig. 5B). Dodatkowo potwierdzono występowanie wzajemnych zależności pomiędzy PKGIα i białkiem VASP, którego ilość zmniejsza się zarówno po transfekcji komórek za pomocą siRNA PKGIα, jaki i po zastosowaniu inhibitora PKG (Fig. 5C).

Nasze wcześniejsze wyniki badań wykazały, że aktywacja PKGIα i wysokie stężenie glukozy wiążą się ze spadkiem poziomu fosforylacji MLC w podocytach[57]. Analiza Western blot potwierdziła, że wysokie stężenie glukozy obniża istotnie ilość formy ufosforylowanej MLC o 21%. W podocytach hodowanych w warunkach hiperglikemicznych, wyciszenie ekspresji genu kodującego PKGIα lub zastosowanie Rp-8-Br-cGMPS zwiększa stopień fosforylacji MLC (Fig. 5D).

W kolejnym etapie badań wykazano, że wysokie stężenie glukozy lub hiperinsulinemia prowadzą do wzrostu stopnia fosforylacji Ser239 białka VASP oraz zwiększają ilość białek VASP i PKGIα w podocytach (Fig. 7). Wyciszenie ekspresji genu kodującego VASP zapobiega tym efektom środowiska hiperglikemicznego/hiperinsulinemii zarówno wobec fosforylacji VASP (Fig. 7A), jak i poziomu białka VASP (Fig. 7B) i ilości białka PKGIα (Fig. 7C). Zbadano także, czy białko VASP jest zaangażowane w regulację stopnia fosforylacji MLC. Podocyty eksponowane na insulinę i/lub wysokie stężenie glukozy charakteryzują się zmniejszoną ilością ufosforylowanej formy MLC. Natomiast zastosowanie siRNA VASP znosi ten efekt (Fig. 7D).

W oparciu o wyniki sugerujące VASP-zależną regulację fosforylacji MLC, zbadano wpływ białka VASP na organizację filamentów aktynowych w podocytach. Na podstawie analizy immunofluorescencyjnej filamentów aktynowych w podocytach hodowanych w warunkach wysokiego stężenia insuliny lub glukozy, stwierdzono wzrost intensywności fluorescencji dla filamentów aktynowych w obszarach przybłonowych. Wyciszenie ekspresji genu kodującego białko VASP prowadzi do zniesienia wpływu insuliny i wysokiego stężenia glukozy na położenie filamentów aktynowych w podocytach (Fig. 8). *Kim i wsp. (2010)* zaobserwowali, że w komórkach mięśni gładkich z wyciszoną ekspresją VASP dochodzi do obniżenia kurczliwości, czemu towarzyszyły zaburzenia w elongacji filamentów aktynowych. Z kolei *Lindsay i wsp. (2007)* zasugerowali, że NO-zależna fosforylacja białka VASP w pozycji Ser239 prowadzi do zmniejszenia ilości filamentów aktynowych w wypustkach komórek nabłonkowych kanalików proksymalnych, zaniku lamellipodii i zaokrąglenia komórek. Otrzymane wyniki sugerują, że w stanach związanych z insulinoopornością może dochodzić do rozregulowania szlaku sygnałowego zależnego od białka VASP, a w konsekwencji do zaburzeń w reorganizacji szkieletu aktynowego podocytów.

W związku z powyższymi wynikami zbadano udział białka VASP w regulacji przepuszczalności monowarstwy podocytów dla albuminy. Badania funkcjonalne potwierdzają stymulujący wpływ insuliny oraz wysokiego stężenia glukozy na

przepuszczalność, podczas gdy po zahamowaniu ekspresji genu kodującego VASP efekt ten jest zniesiony a przepuszczalność dla albuminy przez monowarstwę podocytów maleje (Fig. 9). Wyniki badań przeprowadzonych na komórkach endotelialnych mózgu pokazują, że w warunkach niedotlenienia dochodzi do zwiększenia stopnia fosforylacji białka VASP w pozycji Ser239, co koreluje ze wzrostem przepuszczalności bariery krew-mózg[80]. *Hohenstein i wsp. (2005)* wykazali, że podczas gwałtownie postępującego kłębuszkowego zapalenia nerek dochodzi do zwiększenia ilość białka VASP w podocytach, czemu towarzyszy zmniejszenie ilości podocytów w kłębuszkach.

Powyższe wyniki sugerują, że szlak sygnałowy związany z białkiem VASP może odgrywać istotną rolę w patofizjologii podocytów, tym samym zaburzając funkcjonowanie bariery filtracyjnej w DKD.

6.4. Podsumowanie wyników

Badania przeprowadzone w ramach niniejszej pracy doktorskiej wskazują na istotną rolę kanału jonowego TRPC6 i PKGIα w regulacji funkcji podocytów w warunkach fizjologicznych oraz patofizjologicznych. Otrzymane wyniki dowodzą, że insulina, poprzez TRPC6-zależną oksydacyjną aktywację ścieżki sygnałowej zależnej od PKGIα, zwiększa przepuszczalność izolowanych kłębuszków nerkowych oraz monowarstwy komórek podocytarnych dla albuminy. Jest to nowo scharakteryzowany mechanizm insulinozależnej regulacji przepuszczalności kłębuszkowej bariery filtracyjnej, w którym kluczową rolę odgrywa sygnalizacja wapniowa. Oprócz tego wykazano, że kanał TRPC6, aktywując szlak sygnałowy zależny od AMPKα2, pozytywnie reguluje insulinozależny dokomórkowy transport glukozy i jest zaangażowany w regulację dynamiki filamentów aktynowych w szczurzych podocytach. Badania stanowiące podstawę niniejszej dysertacji, po raz pierwszy pokazują, że kanał TRPC6 odgrywa kluczową rolę w pozytywnej regulacji aktywności AMPK. Ponadto zaproponowano nowy mechanizm łączący sygnalizację wapniową z insulinozależną regulacją dokomórkowego transportu glukozy w szczurzych podocytach.

Eksperymenty przeprowadzone w ramach niniejszej rozprawy doktorskiej potwierdziły, że insulina i wysokie stężenie glukozy zwiększają przepuszczalność monowarstwy podocytów dla albuminy w wyniku aktywacji ścieżki sygnałowej zależnej od PKGIα. Co więcej, jako pierwsi, udowodniliśmy, że białko VASP jest jednym z elementów tego patomechanizmu. Wyniki zaprezentowane w tej pracy sugerują, że w warunkach wysokich stężeń glukozy i/lub insuliny dochodzi do zwiększenia przepuszczalności monowarstwy podocytów dla albuminy w wyniku aktywacji szlaku sygnałowego zależnego od PKGIα, któremu towarzyszy istotne zwiększenie stopnia fosforylacji białka VASP w pozycji Ser239.

Niniejsza dysertacja uzupełnia wiedzę nie tylko na temat roli kanału TRPC6 oraz sygnalizacji wapniowej w regulacji funkcji podocytów, ale wskazuje również nowe potencjalne źródła patomechanizmów powstających w stanach związanych z insulinoopornością, które mogą zaburzać funkcjonowanie kłębuszkowej bariery filtracyjnej. Ponadto wyniki prac ujętych w rozprawie doktorskiej pokazują, że hiperinsulinemia oraz insulinooporność są odpowiedzialne za rozregulowanie szlaków sygnałowych, w tym szlaku sygnałowego PKGIα/VASP, zaburzając prawidłowe funkcjonowanie podocytów.

7. Wnioski

1. TRPC6 pośredniczy w insulinozależnej aktywacji PKGIα, której towarzyszy zwiększona przepuszczalności monowarstwy podocytów oraz izolowanych kłębuszków nerkowych dla albuminy. Uzyskane wyniki sugerują, że szlak sygnałowy TRPC6/PKGIα jest zaangażowany w insulinozależną regulację przepuszczalności kłębuszkowej bariery filtracyjnej. Ponadto powyższe wyniki sugerują, że hiperinsulinemia może prowadzić do zaburzeń w homeostazie wapnia w wyniku zwiększonego napływu jonów Ca^{2+} do komórki, tym samym promując nadmierną produkcję reaktywnych form tlenu, które aktywując PKGIα, przyczyniają się do zwiększania przepuszczalności albuminy przez warstwę podocytów. Mechanizm przedstawiony w niniejszej pracy może tłumaczyć zwiększoną przepuszczalność bariery filtracyjnej w chorobach związanych ze zwiększoną ekspresją kanału TRPC6 oraz zbyt dużymi wewnątrzkomórkowymi stężeniami jonów Ca^{2+} w podocytach.

2. TRPC6 uczestniczy w insulinozależnej regulacji dynamiki aktyny w podocytach poprzez aktywację białek szlaku sygnałowego Rac1/PAK/kofilina, przy czym aktywacja TRPC6 i AMPKα jest niezbędna do pobudzenia szlaku sygnałowego zależnego od Rac1 w odpowiedzi na insulinę. Otrzymane wyniki badań sugerują, że sygnalizacja AMPK i TRPC6 mają istotny wpływ na insulinozależną regulację szlaku sygnałowego Rac1/PAK/kofilina, a w konsekwencji na dynamikę szkieletu aktynowego podocytów. W kontekście zaprezentowanych wyników można przypuszczać, że farmakologiczna kontrola stopnia fosforylacji kofiliny może zapewnić prawidłową dynamikę polimeryzacji aktyny w cukrzycy przebiegającej z insulinoopornością, a także zabezpieczać prawidłowe funkcjonowanie transporterów GLUT4 oraz dokomórkowego transportu glukozy w podocytach.

3. Zahamowanie aktywności TRPC6 prowadzi do zniesienia stymulującego wpływu insuliny na stopień fosforylacji AMPK oraz na dokomórkowy transport glukozy w podocytach. Dlatego też wydaje się, że TRPC6-zależny napływ jonów Ca^{2+}, poprzez aktywację AMPK, jest pozytywnie zaangażowany w insulinozależny dokomórkowy transportu glukozy w podocytach. W związku z tym można przypuszczać, że ograniczenie insulinozależnego transportu glukozy w cukrzycy typu 2 może być

związane z zaburzeniami w homeostazie wapnia, które to mogą prowadzić do zmniejszenia aktywności AMPK.

4. Insulina oraz wysokie stężenie glukozy zwiększają przepuszczalność podocytów dla albuminy na drodze PKGIα-zależnej fosforylacji białka VASP w pozycji Ser239, stanowiącej sygnał dla podocytów do reorganizacji szkieletu aktynowego. Aktywacja szlaku sygnałowego PKGIα/VASP może być częściowo odpowiedzialna za wzrost przepuszczalności bariery filtracyjnej, skutkującej albuminurią u pacjentów cierpiących na hiperglikemię lub hiperinsulinemię. Ponadto powyższe wyniki sugerują, że nadmierna aktywacja PKGIα jest jednym z czynników uczestniczących w rozwoju DKD, a zahamowanie aktywności tej kinazy może być potencjalnym celem terapeutycznym dla pacjentów chorych na DKD.

WNIOSEK KOŃCOWY

Insulina, poprzez TRPC6-zależną aktywację szlaku sygnałowego związanego z PKGIα i AMPKα2/Rac1, reguluje procesy komórkowe kluczowe dla prawidłowego funkcjonowania podocytów. W warunkach patofizjologicznych (wysokie stężenie insuliny i/lub glukozy) aktywacja szlaku sygnałowego PKGIα/VASP skutkuje zwiększeniem przepuszczalności monowarstwy podocytów dla albuminy. Otrzymane wyniki sugerują, że w stanach związanych z insulinoopornością może dochodzić do zaburzenia metabolizmu podocytów w wyniku rozregulowania insulinozależnych szlaków sygnałowych, co z kolei może prowadzić do dysfunkcji podocytów, a w konsekwencji do zmian w strukturze oraz przepuszczalności kłębuszkowej bariery filtracyjnej i rozwoju cukrzycowej choroby nerek.

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9. Kopie publikacji wchodzące w skład zbioru

9.1. Poświadczony przez Bibliotekę IMDiK PAN pięcioletni impact factor (IF) publikacji wchodzących w skład zbioru.

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Insulin increases filtration barrier permeability via TRPC6-dependent activation of PKGIα signaling pathways

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ABSTRACT

Podocytes are dynamic polarized cells on the surface of glomerular capillaries and an essential component of the glomerular filtration barrier. Insulin increases the activation of protein kinase G type Iα (PKGIα) subunits, leading to podocyte dysfunction. In addition, accumulating evidence suggests that TRPC6 channels are crucial mediators of podocyte calcium handling and involved in the regulation of glomerular filtration. Therefore, we investigated whether TRPC6 is involved in the regulation of filtration barrier permeability by insulin *via* the PKGIα-dependent manner.

TRPC channel inhibitor SKF96365 abolished insulin-dependent glomerular albumin permeability and transepithelial albumin flux in cultured rat podocytes. Insulin-evoked albumin permeability across podocyte monolayers was also blocked using TRPC6 siRNA. The effect of insulin on albumin permeability was mimicked by treating podocytes with TRPC channel activator (oleolyl-2-acetyl-sn-glycerol, OAG). Insulin or OAG treatment rapidly increased the superoxide generation through activation of NADH oxidase. TRPC inhibitor SKF96365 or siRNA knockdown of TRPC6 attenuated insulin-dependent increase of ROS production. Furthermore, TRPC inhibitor or downregulation of TRPC6 blocked insulin-induced rearrangement of the actin cytoskeleton and attenuated oxidative activation of PKGIα and changes in the phosphorylation of PKG target proteins MYPT1 and MLC. Moreover insulin regulated the PKGIα interaction with TRPC6 in cultured rat podocytes.

Taken together, our data suggest a key role of TRPC6 channels in the mediation of insulin-dependent activation of PKGIα signaling pathways. Overall, we have identified a potentially important mechanism that may explain disturbances in filtration barrier permeability in many diseases with increased expression of TRPC6 and chronic Ca^{2+} overload.

1. Introduction

Podocytes are continually exposed to transmural hydrostatic pressure, which favors glomerular filtration. These cells express some proteins involved in smooth muscle cell contraction [1,2]. The arrangement of F-actin, myosin, and α-actinin in foot processes has been proposed to facilitate glomerulus adaptation to changes in pressure gradients by modifying the surface area for filtration [3,4]. Podocytes also express receptors for factors that regulate contraction and relaxation, suggesting that podocyte function may be regulated by vasoactive hormones and autocrine/paracrine factors [5,6]. Thus, the hormonal regulation of podocytes affects the size-selectivity of the filtration barrier, and this regulation may be similar to that of smooth muscle cells. Moreover, the size-selective barrier properties of podocytes are regulated by Ca^{2+}-dependent changes in proteins that regulate the cytoskeleton and slit diaphragm (e.g., nephrin, Ca^{2+}-activated K^+ channel (BK_{Ca}), and the transient receptor potential cation channel (TRPC6)) [7].

Protein kinase G type I alpha (PKGIα) is an intracellular target for vasorelaxant factors that is activated in a cGMP-dependent and cGMP-independent manner [8,9]. We previously showed that the PKGIα isoform is expressed in cultured rat podocytes and increases the permeability to albumin [10]. PKG initiates phosphorylation events that lead to several biological actions, including rearrangement of the cytoskeleton, dephosphorylation of MLC, and a reduction of free intracellular calcium and the calcium sensitivity of the contractile apparatus, which destabilizes interactions with the thin filament [11]. A recent study revealed a role of intracellular calcium in the regulation of filtration barrier permeability and PKGIα signaling pathways in cultured rat podocytes [12]. Several investigators have also demonstrated that TRPC6 plays an important role in podocyte function [13–19].

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Table 1
Real-time PCR primers and probe sequences on the TaqMan reaction. dqd — dark quencher dye.

Gene	GenBank accession number	Primer sequences (5′–3′)	Probe sequences (5′–3′)	Product size
TRPC6	NM_053559.1	F: CAGCCGTTTAAAACTCGCTATT R: ACCACGAGGAATTTCACTGC	Yellow555®-CTTCCCCA-dqd	141 bp
β-Actin	NM_031144.3	F: AGGCCCCTCTGAACCCTA R: GGGGTGTTGAAGGTCTCAAA	FAM-CGTGAAAAGATG-dqd	70 bp

Mutations in *TRPC6* have been associated with the human proteinuric kidney disease, focal segmental glomerulosclerosis (FSGS) [20,21]. In this disease, the specific cellular junction structure between podocyte foot processes (slit diaphragm) loses their integrity, disrupting the glomerular filtration barrier. Although over-expression of TRPC6 in the mouse kidney induces proteinuria [22], how the channel activity of mutated TRPC6 is involved in the pathogenesis remains unclear. In contrast, the P112Q mutation increases plasma membrane expression of TRPC6 [20], suggesting that changes in surface expression may contribute to the pathogenesis of the disease. Moreover, an important link between Ca^{2+} and podocyte injury is the cleavage of synaptopodin and proteinuria due to the activation of Ca^{2+}-dependent phosphatase calcineurin. Moreover, over-expression of TRPC6 in podocytes may lead to higher intracellular Ca^{2+} concentrations in the presence of stimuli. The increase in $(Ca^{2+})_{in}$ down-regulates the expression of nephrin and synaptopodin and stimulates RhoA activity, which causes F-actin

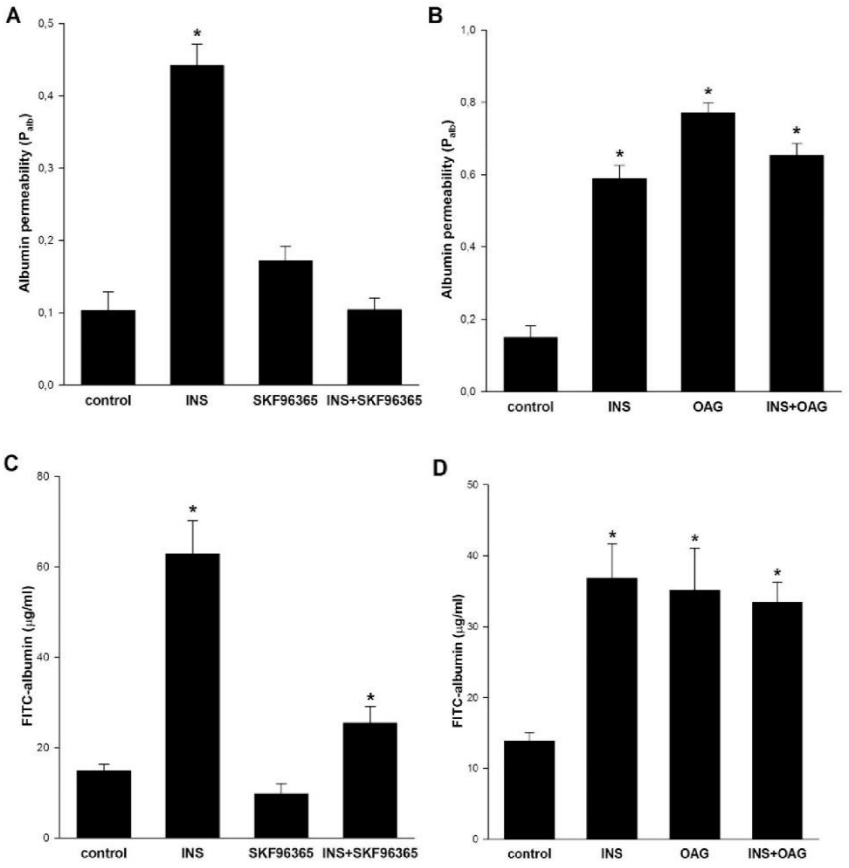


Fig. 1. Insulin increases filtration barrier permeability through TRPC channel activation. (A, B) Glomerular albumin permeability (P_{alb}) was measured in isolated glomeruli exposed to an oncotic gradient with or without insulin (INS, 300 nM, 5 min) and TRPC6 channel inhibitor SKF96365 (10 μM, 15 min pre-incubation) (A) or TRPC channel activator OAG (100 μM, 5 min) (B). The values represent the mean ± SEM (12–18 glomeruli from four rats). *$P < 0.05$ compared to control. (C, D) 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 insulin (INS, 300 nM, 5 min) and TRPC6 channel inhibitor SKF96365 (10 μM, 15 min pre-incubation) (C) or TRPC channel activator OAG (100 μM, 5 min) (D). Results from four to six experiments are shown as mean ± SEM. *$P < 0.05$ compared to untreated podocytes.

derangement and a decrease in foot processes [23]. In addition, sustained TRPC6 activation in cultured podocytes can induce apoptosis [24]. Analogous to other cell types, such as neuronal dendritic spines, dynamic responses in podocytes and their foot processes may be mediated by cytoskeletal elements and Ca^{2+}-dependent processes. TRPC6 is probably a normal part of a compensatory response to mechanical or metabolic stress in podocytes; therefore, inhibiting these channels may actually be counterproductive in glomerular disease. The activation of nitric oxide-cGMP-PKG pathways negatively regulates TRPC6 [25,26] and PKG phosphorylation of TRPC6 at Thr69 prevents TRPC6-mediated Ca^{2+} influx [27]. The precise mechanisms underlying the activation and regulation of TRPC6 remain to be clarified.

Recent studies have shown that insulin can dynamically remodel the actin cytoskeleton of podocytes, which is critically important in maintaining the integrity of the glomerular filtration barrier [28–30]. Actin reorganization results in changes in podocyte structure. Insulin receptor stimulation causes the retraction of podocyte processes. Moreover, in mouse podocytes, specific deletion of the gene encoding the insulin receptor causes a loss of podocyte foot processes [30]. In another study, insulin stimulated the surface expression of BK_{Ca} channel pore-forming subunits (Slo1) [29,31]. These channels require nephrin for steady-state surface expression [32]. Insulin also evokes an increase in the steady-state surface expression of TRPC6 channels in podocytes. This mechanism is dependent on NADPH oxidase-dependent generation of reactive oxygen species (ROS) [14]. This response may be part of a mechanism to maintain the stability of glomerular filtration in the presence of stimuli, such as feeding, that are normally associated with changes in renal hemodynamics. In the present study, we investigated whether TRPC6 is involved in insulin-mediated, PKGIα-dependent regulation of the constriction apparatus and filtration barrier permeability.

2. Material and methods

2.1. Preparation and culture of rat podocytes

All experimental procedures were conducted in accordance with directive 2010/63/EU and approved by the Local Bioethics Commission at the Medical University of Gdansk. We used female Wistar rats weighting 100–120 g. Podocytes were isolated as described previously [28]. Cell phenotype was determined using podocyte-specific antibodies against Wilm's tumor-1 (WT-1) protein (Biotrend Koeln, Germany, Cat. No. BT-53949) and synaptopodin (Progen, Heidelberg, Germany, Cat. No. 65194). Cell viability was determined by detecting lactate dehydrogenase leakage.

2.2. Western blot analysis

Protein supernatants (20 μg) were resolved using a 10% SDS-polyacrylamide gel and electro-transferred to a nitrocellulose membrane. The following primary antibodies were used for Western blot: anti-p-MLC2 (Ser19) (1:400, Cell Signaling, Cat. No. 3671), anti-MLC2 (1:1000, Cell Signaling, Cat. No. 3672), anti-PKGIα (1:400, Santa Cruz Biotechnology, Cat. No. sc-10,335), anti-MYPT1 (1:400, Santa Cruz Biotechnology, Cat. No. sc-25,618), anti-p-MYPT1 (Ser-695) (1:400, Santa Cruz Biotechnology, Cat. No. sc-33,360), anti-TRPC6 (1:1000, Sigma-Aldrich, Cat. No. PRS3897), and anti-actin (1:3000, Sigma-Aldrich, Cat. No. SAB4200248). To detect primary antibodies bound to proteins on the immunoblot, the membrane was incubated for 2 h with the appropriate alkaline phosphatase (AP)-conjugated secondary antibodies. Protein bands were detected using the colorimetric 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) system.

2.3. Immunofluorescence

Podocytes were seeded on coverslips coated with type-I collagen (Becton Dickinson Labware, Beckton, UK) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The cells were fixed in PBS containing 4% formaldehyde for 10 min at room temperature. Next, the coverslips were placed on ice and the cells permeabilized by adding 0.3% Triton-× 100 for 3–4 min before blocking with a PBSB solution (PBS containing 2% FBS, 2% BSA, and 0.2% fish gelatin) for 60 min. After blocking, the cells were incubated with anti-PKGIα and anti-TRPC6 antibodies in PBSB (1:100) at 4 °C for 1 h. Non-specific staining was evaluated by substituting the primary antibodies with PBSB. Next, the cells were washed three times with cold PBS and incubated for 45 min with secondary anti-mouse antibodies conjugated with Alexa-Fluor 488 (1:1000) or anti-goat antibodies (1:1000) conjugated with Alexa Fluor 546. Specimens were imaged using a confocal laser scanning microscope (Leica SP8X) with a 63× oil immersion lens.

The F-actin network was labeled and visualized by fluorescence microscopy (Olympus IX51) as described by Pubill et al. with minor modifications [33]. Digitized fluorescence images of the F-actin network were used to generate fluorescence intensity profiles (from basal membrane to nucleus) using CellSens imaging software. To normalize the fluorescence intensity profiles originating from different cells, we expressed the fluorescence intensity of X-axis pixels at a distance of 1 μm as the percentage of the mean value of the fluorescence intensity for the total X-axis with the cell membrane positioned at point 0. The fluorescence values of each profile were expressed as a percentage of the mean fluorescence value of the corresponding profile.

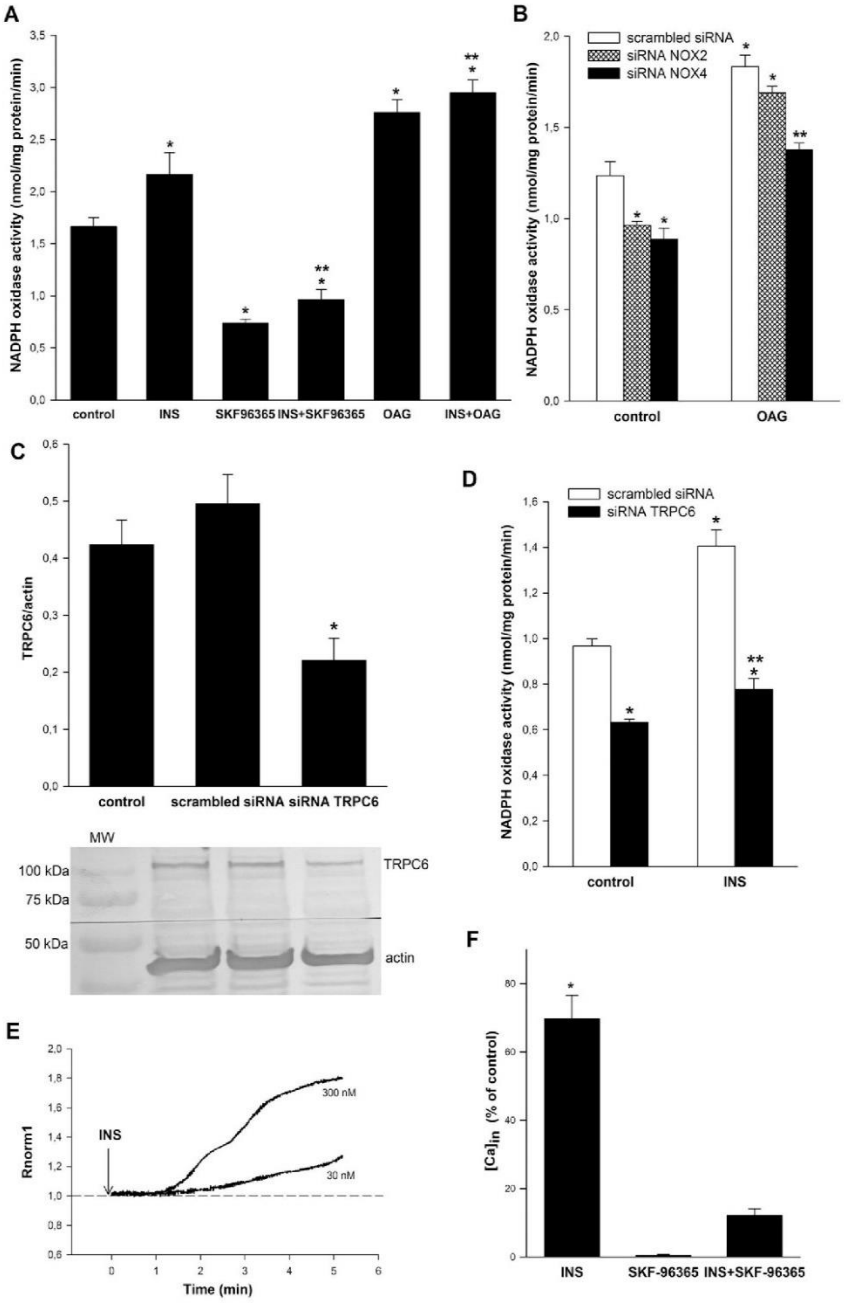
2.4. RNA extraction and real-time PCR analysis

The cultured podocytes were lysed in TRI Reagent (Sigma) and total RNA isolated through chloroform/isopropanol extraction. The RNA quantity was determined by spectrometry and purity assessed by calculating the A260/A280 ratio. A probe was considered pure when the A260/A280 ratio was 1.8–2.2. Next, RNA was treated with DNase (Sigma) and reverse transcription performed using 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 *TRPC6* mRNA level was determined using TaqMan hydrolysis probe (Roche, Tab.1) and gene-specific intron-spanning primers in a Light Cycler 480 (Roche). The results were quantified by the ΔC_t method with β-actin as an internal control. The real-time PCR conditions included a pre-incubation step (10 min at 95 °C), followed by 45 cycles

Fig. 2. The role of TRPC6 channels in the regulation of NADPH oxidase activity in podocytes. (A) The effect of TRPC channel inhibitor (SKF96365, 10 μM, 15 min pre-incubation) and activator (OAG, 100 μM, 5 min) on NADPH oxidase was measured by lucigen-enhanced chemiluminescence. The values represent the mean ± SEM ($n = 4$). $^*P < 0.05$ compared to control, $^{**}P < 0.05$ compared to insulin. (B) The effect of downregulation of NOX2 and NOX4 on OAG-induced NADPH oxidase activity. The values represent the mean ± SEM ($n = 5$). $^*P < 0.05$ compared to control with scrambled siRNA, $^{**}P < 0.05$ compared to scrambled siRNA with OAG. (C) The effect of TRPC6 siRNA and scrambled siRNA on TRPC6 protein expression. Densitometry of the TRPC6 band was normalized to the actin band. The values represent the mean ± SEM ($n = 4$). $^*P < 0.05$ versus controls (transfection with scrambled siRNA or non-transfected podocytes). (D) The role of downregulation of TRPC6 on insulin-dependent activation of NADPH oxidase. The values represent the mean ± SEM ($n = 4$). $^*P < 0.05$ compared to control with scrambled siRNA, $^{**}P < 0.05$ compared to scrambled siRNA with insulin. (E) The effect of insulin on the intracellular calcium concentration. A representative trace for podocytes stimulated with 30 nM or 300 nM insulin. (F) The modification of insulin induces calcium signaling by TRPC channel inhibitor SKF96365. The values represent the mean ± SEM ($n = 8$–12). $^*P < 0.05$ compared to control.

of denaturation (10 s at 95 °C), annealing (30 s at 60 °C), and elongation (1 s at 72 °C). Negative controls included dH_2O instead of cDNA template. The amplified products were electrophoresed in a 2.5% agarose gel, visualized with ethidium bromide, and imaged using the GelDoc-It Imaging System (UVP, Cambridge, UK). The primers used in PCR are provided in Table 1.



2.5. Permeability assay

Trans-epithelial permeability to albumin was evaluated by measuring the diffusion of FITC-labeled BSA (Sigma-Aldrich) across the podocyte monolayer as described previously [10,34]. Rat podocytes (1×10^5 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). The inserts were placed in 24-well plates and the cells allowed to differentiate for 1 week. Before use in experiments, the podocytes were washed twice with PBS and medium on both sides of the insert and the medium 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 the medium in the lower compartment 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 absorbance of FITC-albumin determined by measuring the absorbance at 490 nm using a multimode plate reader (EnSpire PerkinElmer).

2.6. Glomerular permeability to albumin in vitro

The volume response of glomerular capillaries to an oncotic gradient generated by defined concentrations of albumin was measured as described previously [28,35]. Glomeruli were isolated from male Wistar rats (180–220 g). Isolated glomeruli (de-capsulated and devoid of afferent and efferent arterioles [36]) allowed to affix to glass coverslips coated with poly-L-lysine (1 mg/ml) were incubated in medium containing 5% BSA, an inhibitor of TRPC channels (SKF96365, 10 μM, 15 min) or activator of TRPC channels (OAG, 100 μM, 5 min), and insulin (300 nM, 5 min) at 37 °C. Afterwards, the insulin and/or inhibitors 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 (generating no oncotic gradient). The glomerular volume responses were recorded with a videomicroscope (Olympus microscope IX51) before and 1 min after the test reagents were added. The glomerular volume (V) was calculated from the surface area (S) of the glomerulus using the formula $V = 3/4\pi\ (S/\pi)^{3/4}$ in the CellSens Dimension software (Olympus). There is a direct relationship between an increase in glomerular volume (ΔV) calculated as $(V_{final} - V_{initial}) / V_{initial}$ and the oncotic gradient (ΔΠ) applied across the capillary wall. This principle was used to calculate the reflection coefficient of albumin (σ_{alb}), defined as the ratio of ΔVs in the presence (experimental) and absence (control) of an oncotic gradient, or $\sigma_{alb} = \Delta V_{experimental} / \Delta V_{control}$.

The reflection coefficient of albumin was used to calculate the glomerular capillary permeability to albumin (convectional $P_{alb} = 1 - \sigma_{alb}$), which describes the movement of albumin due to water flow. At least 10 glomeruli isolated from three or more rats were studied in each experiment.

2.7. RNA interference and cell transfection

We transfected podocytes with small interfering RNAs (siRNA) that targeted PKGIα, NOX4, NOX2 (Santa Cruz Biotechnology), TRPC6 (Origene) and a control, non-silencing siRNA (scrambled siRNA, negative control). Podocytes were cultured in RPMI 1640, supplemented with 10% FBS. One day before transfection, the culture medium was removed, and cells were cultivated in antibiotic-free RPMI 1640, supplemented with 10% FBS. Then, we transfected cells with the siRNA Transfection Reagent (Santa Cruz Biotechnology), according to the manufacturer's instructions. Briefly, the targeted siRNA, 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. The transfection mixture was then added to the Transfection Medium, mixed gently, and added to the cells. After 7 h, we added growth medium, which contained 2-fold higher FBS and antibiotics. The cells were incubated for an additional 24 h. After transfection, gene silencing was monitored at the protein level with Western blotting.

2.8. Immunoprecipitation

Cell extracts were pre-cleared with mouse IgG plus Protein A/G-PLUS Agarose at 4 °C for 1 h and then incubated with a primary antibody plus Protein A/G-PLUS Agarose at 4 °C overnight. The agarose beads were washed gently with lysis buffer. Proteins were eluted from the beads by adding SDS loading buffer. The mixture was then boiled for 5 min and subjected to Western blot analysis.

2.9. NAD(P)H oxidase assay

NAD(P)H oxidase activity was measured in podocytes using a modified version of the lucigenin-enhanced chemiluminescence method [37,38]. To measure superoxide anion generation, cell homogenates (50 μg protein) were added to PBS buffer containing 1 mM EDTA and 5 μM lucigenin. The assay was initiated by the addition of 100 μM NADPH. Photon emission, in terms of relative light units, was measured every 30 s for 12 min in a FB12 luminometer (Berthold).

2.10. Intracellular calcium measurements

The cytosolic Ca^{2+} response was evaluated using the fluorescent Ca^{2+} indicator Fura-2/AM (Sigma) with minor modifications [12,39]. Podocytes were grown on 35-mm type I collagen coated culture dishes (Becton Dickinson Labware, Beckton, UK), and incubated at 37 °C for 90 min in RPMI 1640 supplemented with 10 μM Fura-2/AM, 0.006% pluronic (Molecular Probes). Cells were then washed and left in 1.5 mM Ca^{2+}/HEPES. Next, the cells were placed in an open Leyden chamber on a 37 °C thermostat-controlled heated stage, and exposed to 340/380 wavelength light using the Olympus xcellence (Olympus) multiple wavelength high-resolution fluorescence microscopy system. Data were presented as fluorescence ratios at 340/380 nm. In each experiment, 10–15 cells were monitored for at least 8 min. Data was demonstrated as changes in intracellular Ca^{2+} and summarized as the percentage change to control of the area under the curve. To equate between experiments the baselines were normalized to 1.

2.11. Measurement of cGMP

A commercially available kit for cGMP (Cyclic GMP EIA Kit, Cayman Chemical Company, Michigan, USA) was used to determine the concentration in lysed podocytes.

2.12. Statistical analysis

Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. Values are reported as the mean ± SEM. Significance was set at $P < 0.05$.

3. Results

3.1. Insulin increases filtration barrier permeability via TRPC channel activation

In the presence of insulin (300 nM, 5 min), significant increases were observed in the albumin permeability (P_{alb}) of isolated glomeruli (0.442 ± 0.030 *vs.* control 0.103 ± 0.026, $n = 14$, $P < 0.001$, Fig. 1A) and the permeability of the podocyte monolayer (from 14.87 ± 1.39 to 62.90 ± 7.32 μg/ml, $n = 8$, $P < 0.05$, Fig. 1C). These effects were prevented by TRPC channel inhibitor SKF96365 (10 μM, 15 min pre-incubation), which resulted in a P_{alb} of 0.104 ± 0.016 ($n = 14$) and a FITC-

albumin permeability of 9.77 ± 2.23 μg/ml ($n = 6$). TRPC channel activator 1-oleoyl-2-acetyl-glycerol (OAG, 100 μM, 5 min) also increased the P_{alb} (0.771 ± 0.028 *vs.* control 0.149 ± 0.033, $n = 12–14$, $P < 0.001$, Fig. 1B) and podocyte permeability (from 13.86 ± 1.22 to 35.12 ± 5.92, $n = 6$, $P < 0.05$, Fig. 1D). Incubation with both insulin and OAG had no additional effect on permeability (Fig. 1B, D).

3.2. The role of TRPC6 channels in the regulation of NADPH oxidase activity in podocytes

We investigated the effect of insulin (300 nM, 5 min), OAG (100 μM, 5 min), and SKF96365 (10 μM, 15 min pre-incubation) on NADPH oxidase activity in rat cultured podocytes. Insulin increased NADPH oxidase activation approximately 30% ($P < 0.05$; Fig. 2A). Incubation with OAG also induced the activation of NADPH oxidase approximately 66% (from 1.66 ± 0.09 to 2.76 ± 0.12 nmol/mg protein/min, $n = 4$, $P < 0.05$). Pre-incubation of podocytes with TRPC channel inhibitor SKF96365 decreased the NADPH oxidase activity by 55% ($P < 0.05$) and abolished the insulin-mediated increase in NADPH oxidase activation. NADPH oxidase is a major source of superoxide anion ($O_2^{\bullet -}$) in podocytes. Recently, we demonstrated that knocking down NOX2 protein expression by 41% or NOX4 protein expression by 40% using siRNA decreases insulin-induced $O_2^{\bullet -}$ generation [28]. In the present study, we showed that downregulation of NOX4 is associated with a significant decrease in OAG-induced activation of NADPH oxidase (25% decrease, from 1.83 ± 0.06 to 1.38 ± 0.04 nmol/mg protein/min, $n = 4$, $P < 0.05$, Fig. 2B). Downregulation of NOX2 did not significantly influence OAG-induced NADPH oxidase activation. To examine the role of TRPC6 in insulin-dependent regulation of superoxide anion generation, we knocked-down TRPC6 protein expression using siRNA (Fig. 2C). TRPC6 protein expression significantly decreased in podocytes transfected with TRPC6 siRNA compared to podocytes transfected with scrambled siRNA (56% decrease, 0.22 ± 0.04 vs. control 0.50 ± 0.05, $n = 4$, $P < 0.05$). Downregulation of TRPC6 channel expression abolished insulin-mediated NADPH oxidase activation (Fig. 2D). We also observed that insulin increased the intracellular calcium concentration, and that this signaling was modified by TRPC channel inhibitor SKF96365 (Fig. 2E, F).

3.3. Intracellular calcium signaling regulates PKGIα-dependent signaling pathways in cultured rat podocytes

Next, we examined the role of TRPC channels in the regulation of PKGIα dimerization in podocytes. Insulin in non-reducing conditions (maleimide 100 mM) increased the oxidative disulfide bond formation of PKGIα (Fig. 3). In non-stimulated podocytes, PKGIα was mainly expressed in its monomeric 75-kD form (86%). In the presence of insulin, the percentage of dimerized (150 kDa) PKGIα increased approximately 190%. This effect was abolished in the presence of TRPC channel inhibitor SKF96365 (10 μM, 15 min pre-incubation, Fig. 3A). In addition, the effect of insulin was mimicked by the TRPC channel activator OAG (100 μM, 5 min). OAG increased the percentage of the dimerized form of PKGIα by 110% (from 0.19 ± 0.04 to 0.40 ± 0.08, $P < 0.05$, Fig. 3B). The combined action of OAG and insulin had no additional effect on PKGIα dimerization.

Activation of the PKGIα isoforms induced MYPT1 phosphorylation at Ser695, which activates MLCP and reduces MLP phosphorylation. We showed that insulin, through the activation of PKGIα, induced MYPT1 phosphorylation, with a subsequent decrease in MLC phosphorylation, in podocytes (Fig. 4). We hypothesized that the activation of TRPC channels may induce a change in MYPT1 and MLC phosphorylation by activating PKGIα. We found that TRPC channel inhibitor SKF96365 abolished the effect of insulin on the phosphorylation of proteins involved in regulating the contractile apparatus of podocytes (Fig. 4A, B). However, TRPC channel activator OAG increased the phosphorylation of MYPT1 (by 58%) and dephosphorylation of MLP (by 52%) (Fig. 4C, D).

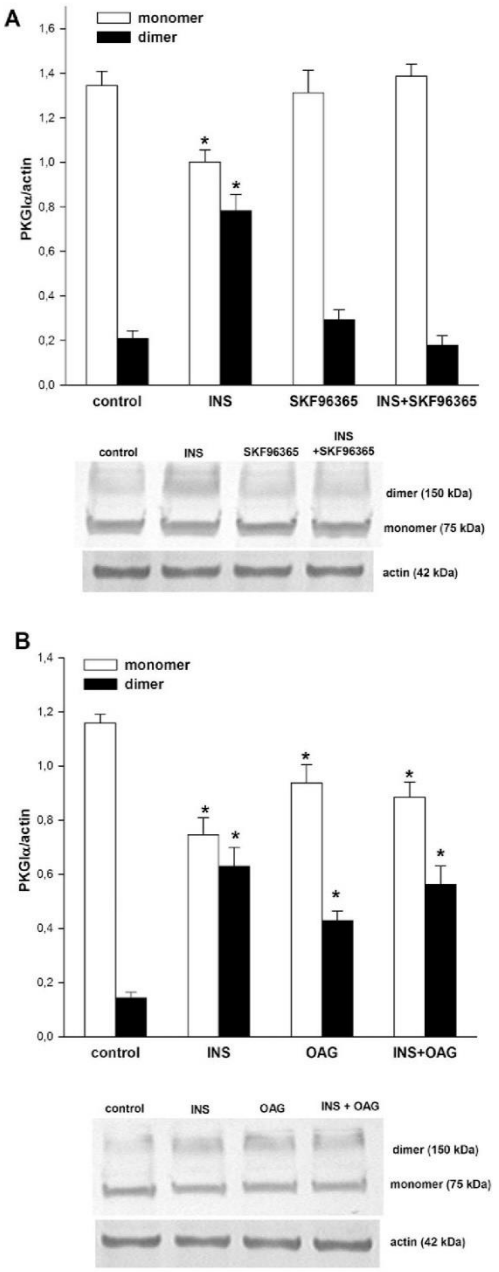


Fig. 3. The role of TRPC channels in insulin-dependent dimerization of protein kinase G type Iα. (A) The effect of TRPC channel inhibitor SKF96365 (10 μM, 15 min pre-incubation) and (B) TRPC channel activator OAG (100 μM, 5 min) in the presence or absence of insulin (INS, 300 nM, 5 min) on PKGIα interprotein disulfide bond formation. Quantitative densitometric analysis of immunoblots is reported as the ratio of PKGIα monomer and dimer band intensities to the actin band intensity. The values represent the mean ± SEM ($n = 4–6$). *$P < 0.05$ compared to appropriate control.

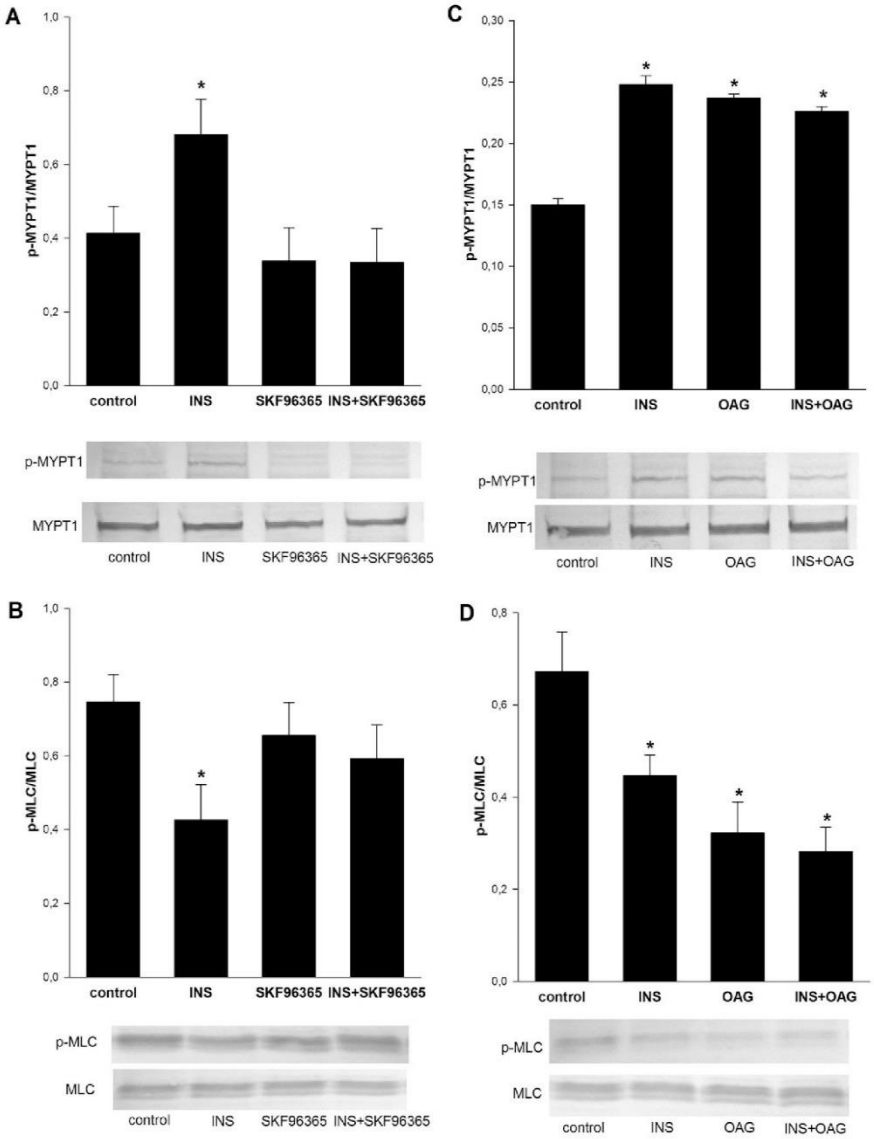


Fig. 4. The role of TRPC channels in insulin-mediated phosphorylation of the myosin-binding subunit of myosin phosphatase 1 (MYPT1) and dephosphorylation of myosin light chain (MLC) in cultured rat podocytes. Podocytes were stimulated with insulin (300 nM, 5 min) in the presence or absence of TRPC channel inhibitor (SKF96365) or activator (OAG). (A, C) Densitometric quantification of the corresponding bands is reported as the ratio of band intensities for p-MYPT1 (Ser695) and MYPT1 and (B, D) p-MLC (Ser19) and MLC. Values represent mean ± SEM ($n = 4–6$). $^*P < 0.05$ compared to control.

We also analyzed the F-actin network in podocytes (Fig. 5). The quantitative analysis confirmed that insulin and OAG directly increased the F-actin immunostaining close to the plasma membrane, and this effect was abolished in the presence of TRPC channel inhibitor SKF96365. We postulated that TRPC channels play essential roles in the regulation of PKGIα-dependent signaling and podocyte contraction.

3.4. The role of insulin in TRPC6 channel interactions with PKGIα and TRPC6 expression in cultured rat podocytes

Recently, several authors demonstrated that TRPC6 forms part of a larger signaling complex of the slit diaphragm and plays a significant role in the regulation of podocyte function [13–19].

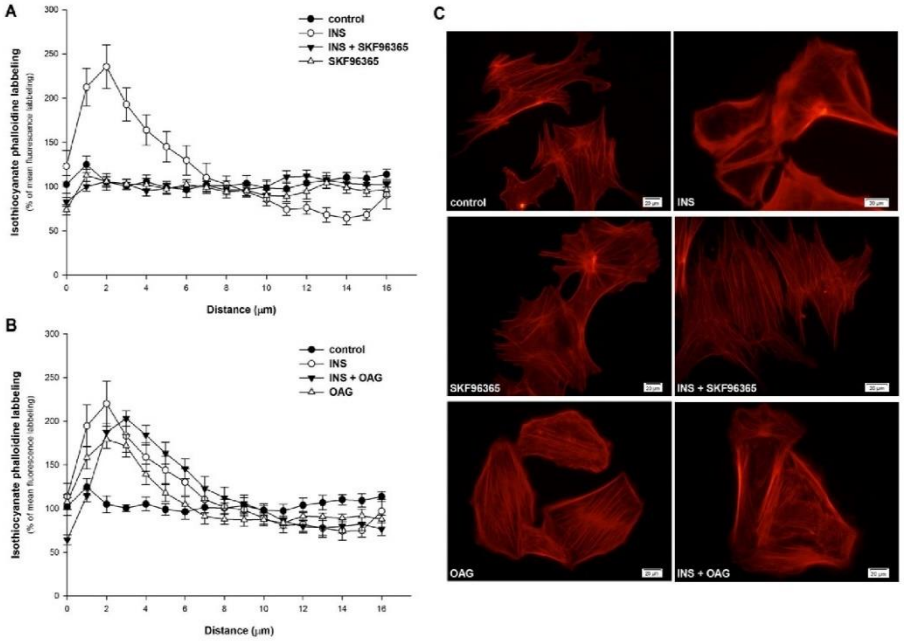


Fig. 5. The effect of TRPC channels in insulin-dependent remodeling of the actin cytoskeleton of podocytes. (A) Cells were grown on coverslips and incubated with INS (300 nM, 5 min) in the presence or absence of TRPC channel inhibitor SKF96365 or (B) TRPC channel activator OAG. The F-actin network was labeled using isothiocyanate phalloidin and visualized by fluorescence microscopy. The digitized fluorescence images of the F-actin network were used to generate fluorescence intensity profiles (from basal membrane to nucleus) using CellSens imaging software. The values represent the mean ± SEM ($n = 8$–10). (C) Representative images.

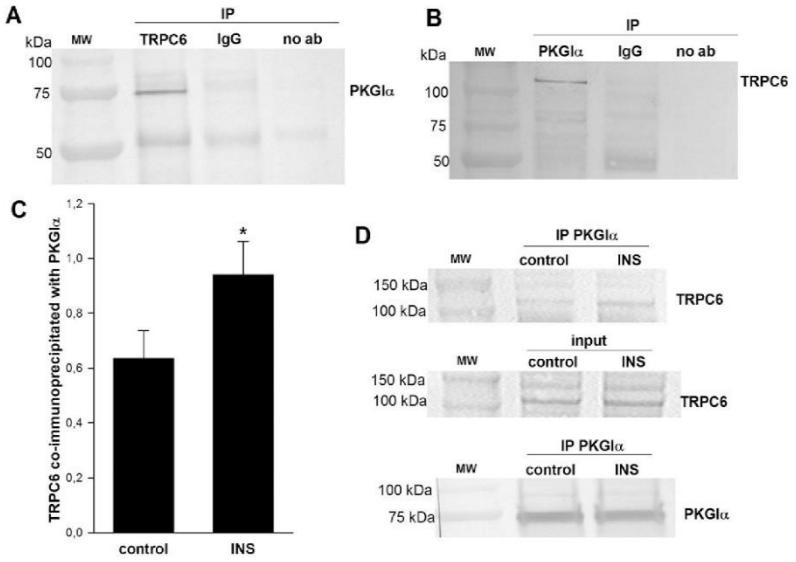


Fig. 6. TRPC6 binds to PKGIα kinase in cultured rat podocytes. (A) Immunoblot showing that PKGIα was associated with the immunoprecipitated TRPC6 in podocyte extracts. (B) Immunoblot showing that TRPC6 was associated with the immunoprecipitated PKGIα. (C) Insulin induced an increase in the amount of TRPC6 that co-immunoprecipitated with PKGIα. Values represent the mean ± SEM ($n = 6$). *$P < 0.05$ compare to control. (D) Representative immunoblots. Input: the line indicates a sample of the original cell lysate (before immunoprecipitation), no ab: no antibody (negative control).

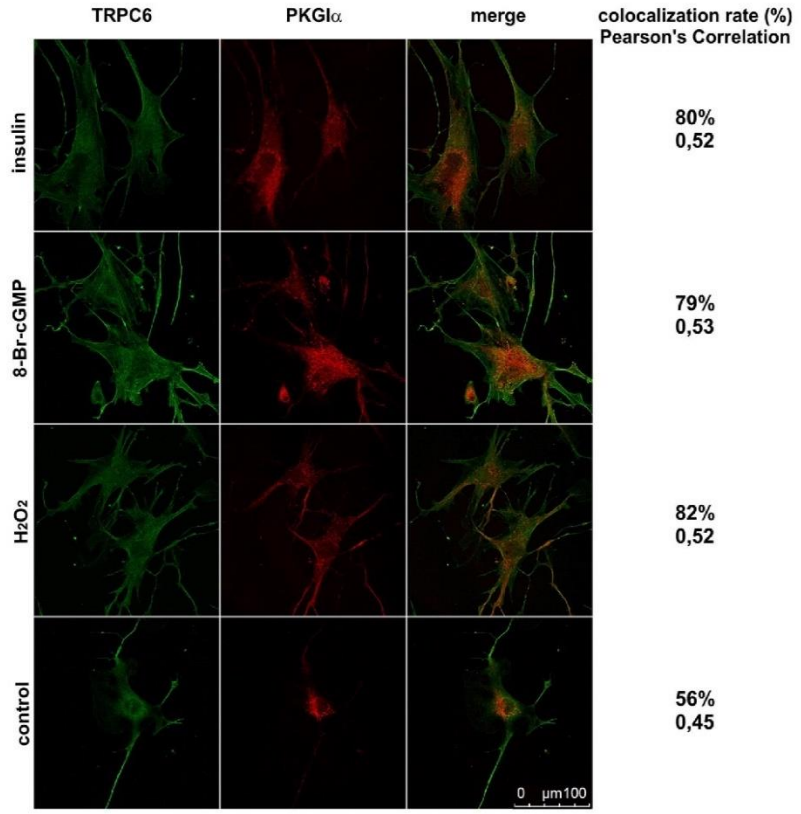


Fig. 7. Activation of PKGIα signaling pathways induced changes in the colocalization of TRPC6 and PKGIα. Rat podocytes seeded onto coverslips were incubated for 5 min with 300 nM insulin, 100 μM 8-Br-cGMP, or 100 μM H_2O_2. The cells were immunostained with anti-TRPC6 and anti-PKGIα antibodies as indicated. Quantitative analysis of protein colocalization was performed using LAS AF 3.3.0 software ($n = 10$). The pixel intensities were quantified and presented as Pearson's correlation and colocalization rate (%).

Here, we addressed whether TRPC6 associates with PKGIα and the role of insulin in this interaction. Podocyte extract mixed with antibody against TRPC6 resulted in the detection of PKGIα in the immunoprecipitate (Fig. 6A). In the reverse procedure, the same interaction was observed (Fig. 6B). We also observed that insulin increased the amount of TRPC6 that co-immunoprecipitated with PKGIα by approximately 47% (from 0.64 ± 0.10 to 0.94 ± 0.12, $n = 7$, $P < 0.05$). The quantitative analysis confirmed that insulin increased the colocalization of TRPC6 and PKGIα proteins (from 56 to 80%, $n = 10$, $P < 0.05$, Fig. 7). The same effect was observed in the presence of PKGI activators (8-Br-cGMP and H_2O_2, 100 μM, 5 min).

We also observed a relationship between TRPC6 gene expression and insulin-dependent regulation of PKGI activity (Fig. 8A). Short-term incubation with insulin (300 nM, 5 min) induced a significant decrease in the *TRPC6* mRNA level (28%, $P < 0.05$, $n = 6$). This effect was prevented by the PKGI inhibitor (Rp-8-cGMPS, 100 μM, 15 min pre-incubation). In addition, short incubation with insulin or PKG activators (8-Br-cGMP and H_2O_2, 100 μM, 5 min) significantly reduced TRPC6 protein (Fig. 8B, ~24%, $P < 0.05$).

3.5. TRPC6 channel-mediated insulin-dependent activation of PKGIα signaling pathways

We further investigated the role of the TRPC6 channel in insulin-dependent regulation of PKGIα signaling pathways by knocking down TRPC6 expression with siRNAs. The basal level of PKGIα protein in podocytes transfected with TRPC6 siRNA decreased approximately 44% (from 0.36 ± 0.03 to 0.20 ± 0.02, $P < 0.05$, Fig. 9A). The effects of insulin on PKGIα oxidative dimerization under non-reducing conditions are shown in Fig. 9B. Insulin increased the percentage of the dimeric form of PKGIα by roughly 105%. Moreover, insulin did not influence the basal cGMP content in podocytes pre-incubated with Zaprinast (10 μM, cGMP-specific phosphodiesterase inhibitor, 40.9 ± 3.1 *vs.* control 49.5 ± 4.0 pmol/mg protein, $n = 4$, $P > 0.05$). Thus, we propose that insulin induced the activation of PKGIα in a ROS-dependent manner.

Downregulation of TRPC6 expression abolished disulfide bond formation in the presence of insulin (Fig. 9A). The activation of PKGIα isoforms by insulin or 8-Br-cGMP (cGMP analog, 100 μM, 5 min) increased MYPT1 phosphorylation and reduced MLC phosphorylation. The effects of insulin were prevented by knocking down TRPC6 protein expression

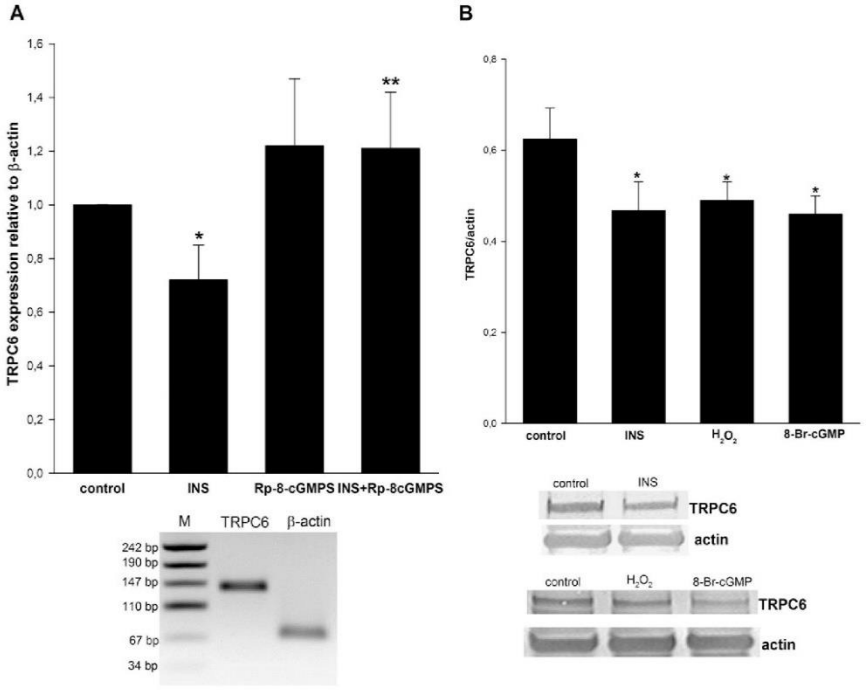


Fig. 8. The role of insulin in TRPC6 gene and protein expression in cultured rat podocytes. (A) Cells were incubated with insulin (300 nM, 5 min) in the presence or absence of PKGI inhibitor (Rp-8-cGMPS, 100 μM, 15 min pre-incubation). TRPC6 and β-actin gene expression profiles were analyzed by RT-PCR. Values represent mean ± SEM ($n = 4$–6), $^{*}P < 0.05$ compared to control, $^{**}P < 0.05$ compared to insulin. The PCR products are shown in the 2.5% agarose gel lines: TRPC6 (141 bp), β-actin (70 bp). (B) Podocytes were stimulated with insulin, 8-Br-cGMP (100 μM, 5 min), and H_2O_2 (100 μM, 5 min). After incubation, cellular proteins were extracted immediately and detected by Western blot. The values represent the mean ± SEM of four independent experiments. $^{*}P < 0.05$ compared to appropriate control.

(Fig. 9C, D). Moreover, TRPC6 downregulation was associated with a diminished effect of 8-Br-cGMP on MYPT1 phosphorylation (20% decrease) and MLC dephosphorylation (21% increase). This effect is likely due to a reduction in the amount of PKGIα protein.

3.6. Insulin increases podocyte albumin permeability via a TRPC6 and PKGIα-dependent mechanism

We investigated the effect of TRPC6 downregulation on podocyte permeability to albumin in the presence of PKGI activators. As expected, insulin and 8-Br-cGMP induced a significant increase in albumin permeability compared to scrambled siRNA control by approximately 186% and 179%, respectively (Fig. 10A). Downregulation of TRPC6 expression abolished the effect of inulin and decreased the effect of 8-Br-cGMP (from 44.36 ± 4.85 to 26.37 ± 2.65 μg/ml, $P < 0.05$) on podocyte permeability.

In the reverse procedure, we knocked down the expression of PKGIα protein. Podocytes transfected with PKGIα siRNA exhibited a significant decrease in PKGIα protein expression (45%, from 0.52 ± 0.04 to 0.27 ± 0.05, $n = 3$, $P < 0.05$) compared to podocytes transfected with scrambled siRNA. Downregulation of PKGIα expression also abolished the effect of insulin and attenuated the influence of TRPC channel activator OAG (from 49.97 ± 4.1 to 32.53 ± 2.51, $P < 0.05$) on podocyte permeability (Fig. 10B). These results suggest that insulin increased the albumin permeability of the podocyte filtration barrier via TRPC6 and PKGIα-dependent activation.

4. Discussion

This study revealed a novel mechanism for insulin-mediated regulation of filtration barrier permeability *via* TRPC6-dependent activation of PKGIα signaling pathways. The proposed mechanism is presented in Fig. 11. First, an insulin-stimulated increase in albumin permeability and actin cytoskeleton reorganization were dependent on TRPC6 channel activation. Second, the oxidative activation of PKGIα signaling pathways was TRPC6-dependent. Third, insulin regulated the PKGIα interaction with TRPC6 in cultured rat podocytes.

Recently, we demonstrated that insulin increases the albumin permeability of both isolated rat glomeruli and podocytes. The present study confirmed that insulin-stimulated ROS generation in podocytes can result in cell membrane localization and dimerization of the cGMP-dependent PKGIα. These observations, coupled with increased albumin permeability of glomeruli isolated from Zucker obese rats (hyperinsulinemia/insulin resistance and oxidative stress model) in which both PKGIα and NOX4 expression are increased, suggest a mechanism by which insulin regulates filtration barrier permeability, which may be dysregulated in diseases [28]. We also showed a relationship between oxidative stress, PKGIα activation, actin reorganization, and changes in the permeability of the podocyte barrier [10,29].

These experiments demonstrate a mechanism in which insulin markedly increases filtration barrier permeability through TRPC6-dependent activation of PKGIα signaling. We used an *ex vivo* system of isolated glomeruli to investigate rapid and subtle changes in albumin permeability without the influence of hemodynamic (changes in

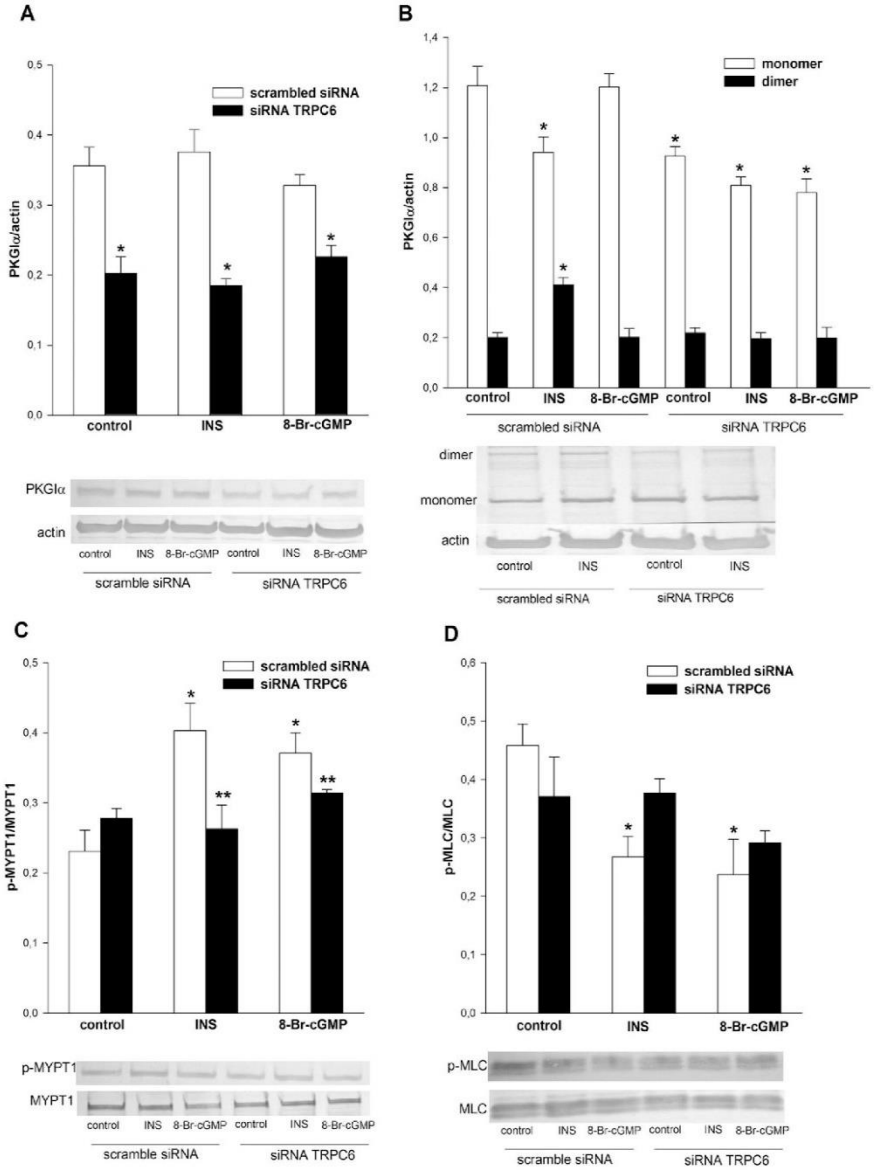


Fig. 9. TRPC6 channel-mediated insulin-dependent activation of PKGIα signaling pathways. (A) The effects of downregulation of TRPC6 on PKGIα protein expression, (B) PKGIα dimerization, (C) MYPT1 phosphorylation, and (D) MLC phosphorylation in podocytes. Densitometric analysis of the corresponding bands is reported as the ratio of band intensities. The values represent the mean ± SEM of four independent experiments. *$P < 0.05$ compared to appropriate control, **$P < 0.05$ compared to appropriate control with scrambled siRNA.

glomerular filtration rate) or circulating factors (cytokines). We postulated that insulin increases glomerular and podocyte permeability via TRPC-mediated Ca^{2+} entry. The inhibition of TRPC channels abolished the effect of insulin on albumin permeability. Moreover, downregulation of TRPC6 expression abolished the effect of inulin on the albumin permeability of the podocyte monolayer. This indicates that TRPC6 activation is necessary for insulin-dependent regulation of filtration barrier permeability. We also showed that podocyte permeability changed in the absence of PKGI activator, and this effect was inhibited after knocking down TRPC6 expression. In the reverse procedure,

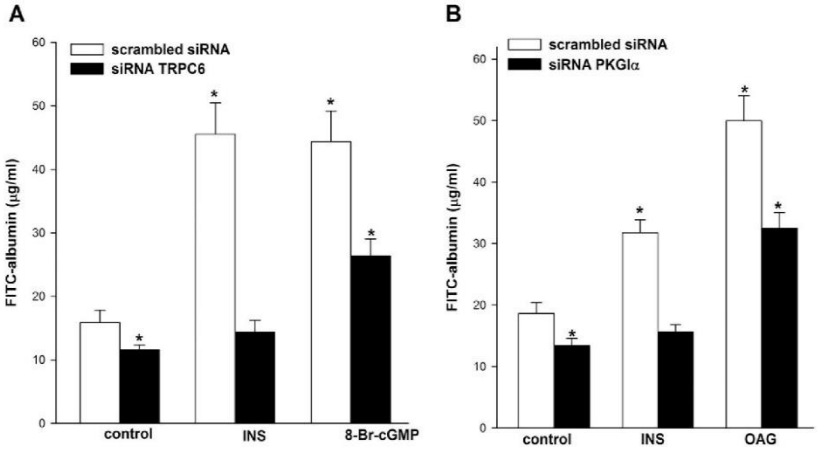


Fig. 10. The influence of a mutual interaction between TRPC6 and PKGIα on albumin permeability across a podocyte monolayer. Small-interfering RNA (siRNA) targeting TRPC6 and PKGIα were introduced into rat podocyte monolayers. Controls were run with scrambled siRNA. The values represent the mean ± SEM of four independent experiments. *$P < 0.05$ compared to control.

downregulation of PKGIα expression abolished the effect of insulin and attenuated the influence of TRPC channel activator OAG on podocyte permeability. These results suggest that insulin led to increased the albumin permeability of the podocyte filtration barrier via TRPC6 and PKGIα-dependent activation. Recently, we demonstrated that this effect is also dependent on large-conductance BK_{Ca} mobilization [29].

This study is the first to show that PKGIα co-immunoprecipitates with TRPC6 in podocytes. Recently, we showed that PKGIα interacts with the Slo1 subunits of BK_{Ca} and plays a role in regulating the surface expression of the Slo1 subunit. BK_{Ca} has been shown to interact with TRPC6 channels and other slit diaphragm proteins, playing an important role in podocyte signaling [40]. Furthermore, co-immunoprecipitation and glutathione-S-transferase pull-down assays have shown that the Slo1 subunits of BK_{Ca} can bind to TRPC6 in cultured mouse podocytes. This colocalization may allow them to serve as a source of Ca^{2+} for the activation of BK_{Ca} [41]. The current study demonstrated that TRPC6-dependent influx of Ca^{2+} induced the oxidative activation of PKGIα. We hypothesized that the interaction of PKGIα with TRPC6 and BK_{Ca} in podocytes is part of a larger complex, which could be part of a mechanism that maintains the stability of glomerular filtration in the face of various stimuli, such as insulin.

Furthermore, we demonstrated that short incubation with insulin decreased TRPC6 gene expression, which was prevented by PKGI inhibition. The TRPC6 protein level was also decreased after incubating podocytes with insulin or activating the PKGI signaling pathway (hydrogen peroxide, 8-Br-cGMP). Another authors also demonstrated that TRPC6 expression was significantly reduced by hydrogen peroxide or by activators of GMP/PKG-dependent signaling [26,42]. This may indicate an adaptive mechanism to counteract the accumulation of intracellular calcium and decrease podocytes contraction. How PKGIα suppresses the expression of TRPC6 is currently unknown. Probably, it could be through a direct modulation by phosphorylating specific transcription factors that bind the promoter region of the TRPC6 gene or through an direct mechanism by phosphorylating one or more downstream molecules. One of the study provided evidence that nuclear factor κB is a key molecule downstream of hydrogen peroxide/PKC in the cascade of TRPC6 gene regulation in the kidney [42]. Another study showed that the activation of PKGI-dependent pathways negatively regulates TRPC6 activity. TRPC6 channels have also been shown to be negatively regulated by the NO/cGMP/PKG pathway through Thr69 phosphorylation of the N-terminus [25,27]. Moreover, both pharmacological and genetic agonists of PKG signaling promote TRPC6 phosphorylation and attenuate angiotensin II-induced podocyte dysmotility [43]. All of the findings suggest that effective activation of PKG signaling may be beneficial as a potent negative modulator of TRPC6 conductance in various glomerular diseases.

Recently, we demonstrated that insulin induces the oxidative activation of PKGIα in a manner dependent on the NOX4-dependent generation of ROS [28]. In the present study, the oxidative activation of PKGIα was TRPC6-dependent. We observed an increase in NADPH oxidase activity and the dimerization of PKGIα in the presence of OAG. The effect of insulin on NADPH oxidase activity and oxidative activation of PKGIα signaling was attenuated after TRPC6 knockdown. Moreover, the OAG-induced activation of NADPH oxidase was mainly NOX4-dependent in cultured rat podocytes. Other authors have observed that insulin evokes a marked and rapid increase in the surface expression of TRPC6 channels and NOX4 subunit in podocytes. The effect of insulin on TRPC6 was reduced by NOX4 knockdown, and the effect of insulin on TRPC6 was mimicked by treating podocytes with hydrogen peroxide [14]. Oxidative activation occurs with many TRPC channel members but was first observed for TRPC6 in HEK293 cells, in which H_2O_2 significantly stimulates Ca^{2+} entry in a dose-dependent manner [44]. ROS generation also underlies increased TRPC6 channel activation in podocytes in the presence of high glucose [15], puromycin aminonucleoside [45], and angiotensin II [46]. Furthermore, the formation of TRPC6-NOX2 complexes has been shown to occur only in the presence of podocin, and that the DAG analog stimulates the assembly of these complexes on the cell surface. Moreover the authors showed the loss of DAG-evoked ROS production after podocin knockdown [47]. The our study demonstrated that downregulation of NOX2 did not influence on OAG-dependent production of ROS but decrease the insulin-dependent activation of NADPH oxidase [28]. These data showed that insulin and OAG activates TRPC6 channels *via* different signaling pathways, but this needs to be further evaluated. All of these results indicate that ROS lead to increased Ca^{2+} influx through all models of TRPC6 activation.

The present study also demonstrated that insulin-dependent rearrangement of the cytoskeleton and MLC dephosphorylation is TRPC6 and PKGIα-dependent. Insulin could dynamically remodel the actin cytoskeleton of podocytes, and this is critically important in maintaining

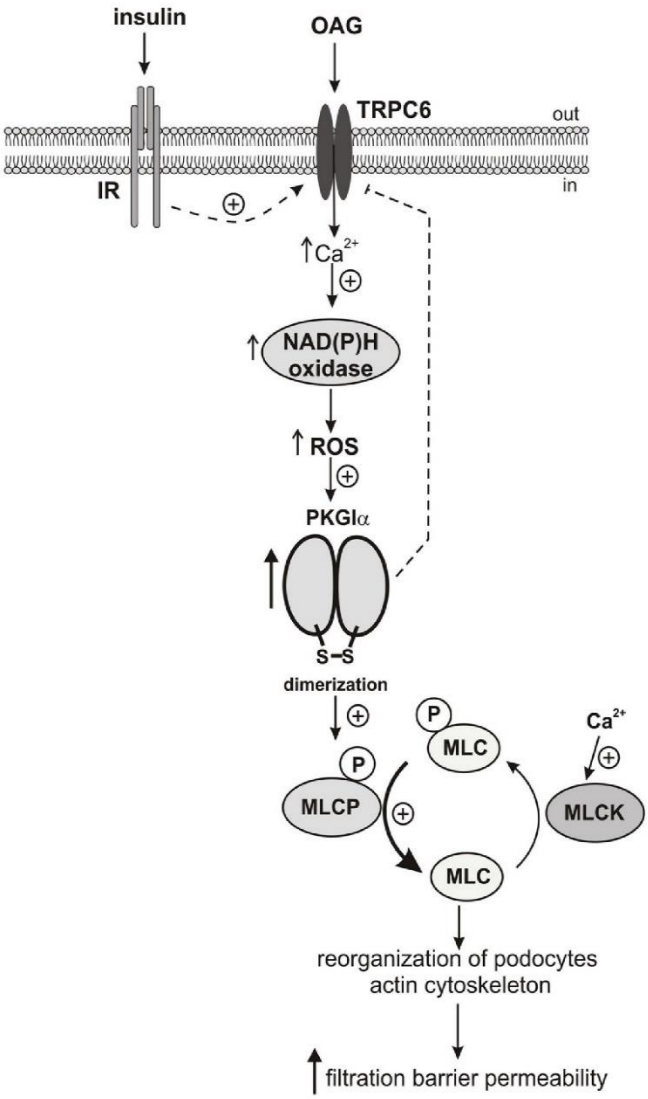


Fig. 11. The suggested mechanism for insulin and TRPC6-dependent activation of PKGIα signaling in cultured rat podocytes.

the integrity of the glomerular filtration barrier. Actin reorganization results in changes in the podocyte structure [28,30]. Other groups have suggested a role of insulin in the control of podocyte contractility, which may contribute to glomerular permeability [14,31].

In summary, this study demonstrated that insulin rapidly stimulates an increase in filtration barrier permeability through TRPC6-dependent activation of PKGIα signaling pathways. This mechanism may explain disturbances in filtration barrier permeability in many diseases accompanied by the increased expression of TRPC6 and chronic Ca^{2+} overload.

Transparency document

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

Acknowledgments

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Original Paper

The TRPC6-AMPK Pathway is Involved in Insulin-Dependent Cytoskeleton Reorganization and Glucose Uptake in Cultured Rat Podocytes

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Key words
AMP-activated protein kinase • Calcium • Cytoskeleton • Glucose uptake • Insulin • Rho family of GTPases

Abstract
Background/Aims: Podocytes are dynamic polarized cells on the surface of glomerular capillaries that are an essential part of the glomerular filtration barrier. 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 are involved in regulating glomerular filtration. Here we investigated whether the AMPK-TRPC6 pathway is involved in insulin-dependent cytoskeleton reorganization and glucose uptake in cultured rat podocytes. ***Methods:*** Western blot and immunofluorescence analysis confirmed AMPKα and TRPC6 expression, the phosphorylation of proteins associated with actin cytoskeleton reorganization (PAK, rac1, and cofilin), and the expression of insulin signaling proteins (Akt, Insulin receptor). Coimmunoprecipitation and immunofluorescence results demonstrated AMPKα/TRPC6 interaction. To ask whether TRPC6 is involved in the insulin regulation of glucose transport, we measured insulin-dependent (1, 2-^{3}H)-deoxy-D-glucose uptake into podocytes after reducing TRPC6 activity pharmacologically and biochemically (TRPC6 siRNA). ***Results:*** The results suggested a key role for the TRPC6 channel in the mediation of insulin-dependent activation of AMPKα2 and glucose uptake. Moreover, AMPK and TRPC6 activation were required to stimulate the Rac1 signaling pathway. ***Conclusion:*** These results suggest a potentially important new mechanism that regulates glucose transport in podocytes and that could be injurious during diabetes.

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KARGER

Introduction

The impairment of insulin signaling and insulin pathways in skeletal muscle, fat tissue, and the liver is central to the development of type 2 diabetes. The resulting dysregulation of glucose and fat metabolism leads to kidney failure and/or to cardiovascular complications. Indeed, the majority of patients with albuminuria and end-stage renal failure in Western countries show abnormalities in insulin production or in insulin effectiveness [1, 2].

Podocytes are uniquely sensitive to insulin and have similarities to skeletal muscle and fat cells with respect to insulin-stimulated glucose uptake kinetics and the expression of glucose transporters (GLUTs) [3, 4]. Recent studies have shown that insulin can dynamically remodel the actin cytoskeleton of podocytes and that this is critically important for maintaining the integrity of the glomerular filtration barrier. Actin reorganization leads to changes in podocyte structure, and insulin receptor stimulation causes the retraction of podocyte processes [5]. Some groups have suggested that insulin plays a role in the control of podocyte contractility, which may contribute to glomerular permeability [6-9]. We recently demonstrated that insulin remodels the actin cytoskeleton and increases the albumin permeability of both isolated rat glomeruli and podocytes; we further showed that the underlying mechanism is calcium-dependent [10, 11]. Moreover, in mouse podocytes, insulin increases the steady-state cell surface expression of TRPC6 channels [9].

Overexpression or gain of function of the TRPC6 channel can drive podocyte effacement, and TRPC6 overexpression in the mouse kidney induces proteinuria [12]. An increase in intracellular calcium downregulates the expression of nephrin and synaptopodin and stimulates RhoA activity, which in turn causes F-actin derangement and a decrease in foot processes [13]. Furthermore, TRPC6 gene mutations are linked to human proteinuric kidney disease, focal segmental glomerulosclerosis, and loss of podocytes [14].

We postulated that dynamic responses in podocytes and in their foot processes are mediated by cytoskeletal elements and by Ca^{2+}-dependent processes. However, the means by which proteins regulate actin dynamics in podocyte foot processes are not fully understood, especially in diabetes. In many types of glomerular diseases, the integrity of the actin cytoskeleton is altered in podocytes, indicating that proper organization and regulation of the actin cytoskeleton are essential for podocyte structure and function [15].

AMP-activated protein kinase (AMPK) activity appears to positively regulate insulin-dependent glucose uptake and insulin signaling [16, 17]; however, AMPK activity is suppressed in disorders associated with insulin resistance [4, 18]. The AMPK is composed of three subunits, one catalytic, termed α, and two regulatory, termed β and γ. The activation of AMPK requires the phosphorylation of threonine 172 (Thr172) within the catalytic α subunit by upstream kinases, namely the Ca^{2+}/calmodulin-dependent kinase kinase-β (CaMKK-β) and/or the LKB1–STRAD–MO25 complex [19]. Hypoxia and contractile activity also activate AMPK. In addition to the potential requirement for AMPK activity, normal regulation of contraction- and exercise-stimulated glucose uptake also requires the Rho GTPase Rac1 [20, 21], which is activated by insulin and which induces actin cytoskeleton remodeling at the plasma membrane in skeletal muscle cells [22]. Rac1 mediates this process by inducing cortical F-actin remodeling, which involves the recruitment of actin regulatory proteins such as cofilin and Arp2/3 to the actin filaments [23]. In addition, Rac1 signals p21-activated kinase 1 (PAK1) in skeletal muscle and facilitates PAK1 phosphorylation in response to insulin [24]. Insulin-stimulated PAK1 activation is decreased in human skeletal muscle in both acute (intralipid infusion) and chronic (obesity and type 2 diabetes) insulin resistant states [22], suggesting that PAK1 is a required element for maintaining euglycemia and insulin sensitivity. These findings suggest that Rac1 and downstream signaling to the actin cytoskeleton constitute an important dysfunctional pathway in insulin-resistant states. This mechanism not be recognized in podocytes.

In the present study, we investigated whether the TRPC6-AMPK pathway is involved in insulin-mediated cytoskeleton reorganization and glucose uptake. Our results identified a potentially important new mechanism that may be injurious to podocytes in diabetes and consequently interfere with the intracellular transport of glucose.

Materials and Methods

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 at the Medical University of Gdansk.

We used female Wistar rats weighting 100–120 g. Podocytes were isolated as described previously [25]. Experiments were conducted using podocytes cultivated for 12–20 days. Cell phenotypes were established using podocyte-specific antibodies against Wilms tumor-1 protein (WT-1; Biotrend Koeln, Germany) and synaptopodin (Progen, Heidelberg, Germany).

Western blot analysis

To obtain podocyte lysate, 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 in the supernatant (12 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-p-insulin Rβ (Tyr1150-1151) (1:200, Santa Cruz Biotechnology), anti-insulin Rβ (1:200, Santa Cruz Biotechnology), anti-p-Akt1/2/3 (Ser473) (1:400, Santa Cruz Biotechnology), anti-Akt1/2/3 (1:400, Santa Cruz Biotechnology), anti-TRPC6 (1:1000, Sigma-Aldrich), anti-p-PAK 1/2/3 (Thr 423/402/421) (1:800, Sigma-Aldrich), anti-p-PAK1/2/3 (Ser144/141/139), 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), 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 using the colorimetric 5-bromo-4-chloro-3-indolylphasphate/nitroblue tetrazolium (BCIP/NBT) system.

Measurement of glucose uptake

Glucose uptake was measured as described previously [4] by the addition of 1 µCi/well of (1, 2-^{3}H)-deoxy-D-glucose diluted in non-radioactive glucose to a final concentration of 50 µM with or without 300 nM insulin. After incubation for 3 min, intracellular and extracellular radioactivity was measured by liquid scintillation counting using a MicroBeta2 Microplate Counter (Perkin Elmer, Waltham, MA, USA).

RNA interference and cell transfection

Podocytes were transfected with small interfering RNAs (siRNAs) that targeted TRPC6 (OriGene), AMPKα1, AMPKα2 (Santa Cruz Biotechnology) or with control, non-silencing siRNA (scrambled siRNA, negative control) (OriGene or 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 siRNAs using siRNA Transfection Reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions. Briefly, the targeted siRNA 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 grow medium supplemented with 2× higher concentrations of FBS and antibiotics. The podocytes were incubated for an additional 24 h. After transfection, gene silencing was checked at the protein level by Western blotting.

Immunofluorescence

Podocytes were seeded on 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 and 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. The primary antibodies 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 using Alexa Fluor 594 phalloidin (1:200). Specimens were imaged using a confocal laser scanning microscope (Leica SP8X) with a 63× oil immersion lens.

Immunoprecipitation

Cell extracts were pre-cleared with mouse IgG plus Protein A/G-PLUS Agarose at 4°C for 1 h and then incubated with a primary antibody plus Protein A/G-PLUS Agarose at 4°C overnight. The agarose beads were washed gently with lysis buffer. Proteins were eluted from the beads by adding SDS loading buffer. The mixture was then boiled for 5 min and subjected to Western blot analysis.

Rac1 activity assay

Rac1 activation was measurement in the supernatant using a commercially available G-LISA Rac1 Activation Assay Biochem Kit (BK128; Cytoskeleton, Inc.)

Statistical analysis

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

Results

Intracellular calcium signaling regulates insulin signal transduction in cultured rat podocytes

First, we investigated the effects of intracellular calcium signaling on the phosphorylation of proteins involved in insulin signal transduction in cultured rat podocytes (Fig. 1). Insulin treatment (300 nM, 5 min) increased the phosphorylation of the insulin receptor (IR) by 262% (from 0.34±0.07 to 1.23±0.24, n=4, $P<0.05$) and increased the phosphorylation of Akt by 32% (from 1.69±0.04 to 2.24±0.03, n=4, $P<0.05$). Incubation of the podocytes with inhibitors of calcium extrusion, namely La^{3+} (1 mM), caloxin2A1 (CX, 0.3 mM), or CB-DMB (50 μM) had no effect on IR and Akt phosphorylation. Insulin treatment of podocytes in the presence of all of the inhibitors of calcium extrusion increased the phosphorylation of IR and Akt to similar extents.

Next, we evaluated the effect of a TRPC inhibitor (SKF96365, 100 μM, 15 min preincubation) on insulin signal transduction in the cultured podocytes. SKF96365 did not affect IR phosphorylation, but it blocked the effect of insulin on Akt phosphorylation (1.47±0.11 vs. control 1.46±0.18, n=4, Fig.1). We then evaluated the role of TRPC6 on insulin signal transduction in podocytes by knocking down TRPC6 expression using siRNA (Fig. 2A). There was a significant decrease in TRRP6 protein expression in podocytes transfected with TRPC6 siRNA compared to podocytes transfected with the control scrambled siRNA (55% decrease, 0.221±0.039 vs. control 0.490±0.013, n=4, $P<0.05$). Downregulation of TRPC6 expression abolished insulin-induced Akt phosphorylation without affecting IR phosphorylation (Fig. 2B, 2C).

Intracellular calcium signaling regulates the insulin-dependent activation of AMPK kinase and glucose uptake in cultured rat podocytes

AMP-activated protein kinase (AMPK) is a major regulator of insulin-dependent glucose uptake and insulin signaling in podocytes [4]. We found that insulin (300 nM, 5 min) induced the phosphorylation of the AMPKα subunit (0.799±0.081 vs. control 0.569±0.042, n=4, $P<0.05$, Fig. 3A). We hypothesized that insulin might increase AMPKα phosphorylation by activating TRPC channels. Indeed, preincubation of podocytes with the TRPC channel inhibitor SKF96365 (100 μM, 15 min preincubation) abolished the effect of insulin on AMPK phosphorylation (Fig. 3A). The same effect was observed after downregulation of TRPC6 expression in podocytes (Fig. 3B). We then demonstrated that podocytes were insulin-sensitive and that insulin stimulation increased glucose uptake by about 40% (Fig. 3C). To determine the effect of TRPC channels on insulin-dependent glucose uptake, we evaluated the effect of SKF96365 (Fig. 3C) or specific TRPC6 inhibitor SAR7334 (1 μM, 15 min, Fig. 3D) and downregulation of TRPC6 expression (Fig. 3E) and found that all blocked insulin-dependent glucose uptake. We therefore postulated that TRPC6 channels play an essential role in the regulation of insulin-dependent signaling and thus in the regulation of glucose uptake in podocytes.

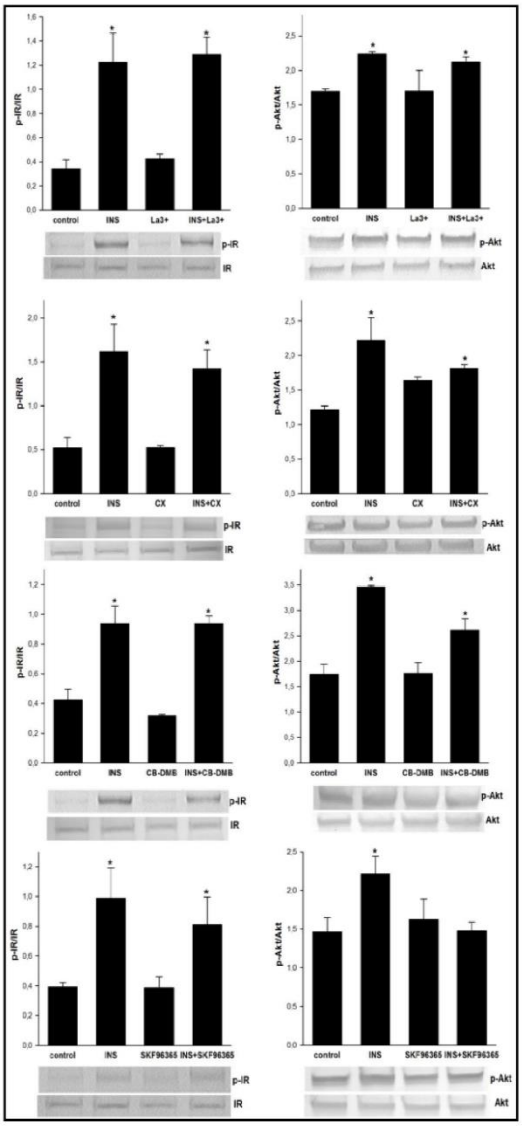

Fig. 1. The effect of intracellular calcium signaling on the phosphorylation of proteins involved in insulin signal transduction in cultured rat podocytes. Podocytes were incubated with or without insulin (300 nM, 5 min) and the indicated calcium signaling inhibitors. Cell lysates were analyzed by immunoblotting using anti-IR, anti-p-IR (Tyr1150/1151), anti-Akt, and anti-p-Akt (Ser473) antibodies. Values are reported as the means±SEMs of four independent experiments. *P<0.05 compared to control.

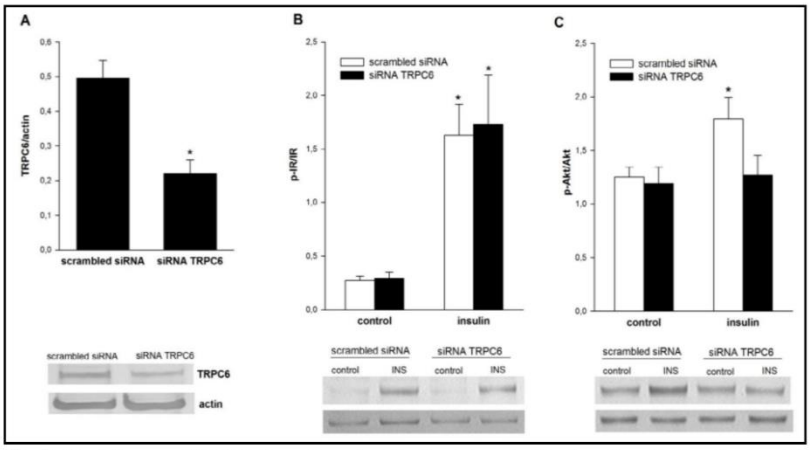

Fig. 2. The influence of TRPC6 channels on insulin signal transduction in cultured rat podocytes. (A) The effect of TRPC6 siRNA on TRPC6 expression. Densitometric measurements of the TRPC6 bands were normalized to the actin band. The influence of TRPC6 downregulation on insulin-dependent phosphorylation of insulin receptor (B) and kinase Akt (C). Values are reported as the means±SEMs of four to six independent experiments. *P<0.05 compared to appropriate control.

The role of insulin in TRPC6 channel interactions with AMPKα subunits in cultured rat podocytes

Many groups have shown that TRPC6 is part of a larger signaling complex in the slit diaphragm and that this complex plays an important role in the regulation of podocyte function. We investigated whether AMPKα subunits associate with TRPC6 as well as the possible role of insulin in this interaction. After mixing podocyte extract with antibodies against AMPKα1 or AMPKα2 subunits, TRPC6 was detected in both immunoprecipitates. Similarly, antibodies to TRPC6 coimmunoprecipitated AMPKα1 and AMPKα2 (Fig. 4A). We also found that insulin increased the amount of TRPC6 that co-immunoprecipitated with AMPKα2 subunits by approximately 45% (from 0.579±0.064 to 0.838±0.055, n=3, $P<0.05$, Fig. 4B). The quantitative analysis confirmed that insulin increased the colocalization of TRPC6 with AMPKα2 subunits (from 57.5% to 72%, n=8, $P<0.05$, Fig. 4D). Insulin did not increase the level of TRPC6 that colocalized with AMPKα1 subunits.

The role of TRPC6 and AMPK in insulin-dependent regulation of Rac1 activity in podocytes

The small GTPase Rac1 is a major regulator of actin remodeling, and cytoskeletal rearrangement is required for GLUT4 translocation in response to insulin [26]. Because we found that inhibition of TRPC6 decreased insulin-stimulated glucose uptake, we hypothesized that this might be due to impaired Rac1-dependent regulation of the actin cytoskeleton. Rac1 is activated when bound to GTP. We found that insulin induces activation of Rac1 by 36% compared to control (1.34±0.02 vs. control 0.86±0.02, n=4, $P<0.05$). Moreover, preincubation of podocytes with TRPC inhibitor (SKF96365, 100 μM, 15 min) attenuated the effect of insulin on Rac1 activity (Fig. 5A). To determine the influence of AMPK on Rac1, we modified AMPK kinase activity using the AMPK activator metformin (2 mM) and the AMPK inhibitor compound C (100 μM) (Fig. 5B). We found that metformin increased glucose uptake by 37% (0.993±0.063 vs. control 0.725±0.048, $P<0.05$, Fig. 5C). However, the AMPK inhibitor compound C decreased glucose uptake to 0.617±0.029 ($P<0.05$, Fig. 5C).

Similar to insulin, metformin also increased Rac1 activity by 39% (1.21±0.14 vs. control 0.87±0.06, n=6, $P<0.05$, Fig. 5D) and Rac1 serine 71 phosphorylation by 25% (1.53±0.07 vs. control 1.22±0.09, n=5, $P<0.05$, Fig. 5E). Inhibiting AMPK activity with compound C decreased Rac1-GTP binding by 29% and Rac1 phosphorylation by 20%.

We then evaluated the roles of the AMPKα1 and AMPKα2 subunits in the regulation of Rac1 activity. We knocked down AMPKα1 and AMPKα2 expression using siRNA (Fig. 6A, B). Only downregulation of AMPKα2 expression decreased Rac1 phosphorylation in the control (by 21%, $P<0.05$, Fig. 6D) and in the presence of metformin (25%, $P<0.05$, Fig. 6D). These data suggest that activation of both TRPC6 and AMPKα2 are necessary to activate Rac1 in cultured rat podocytes.

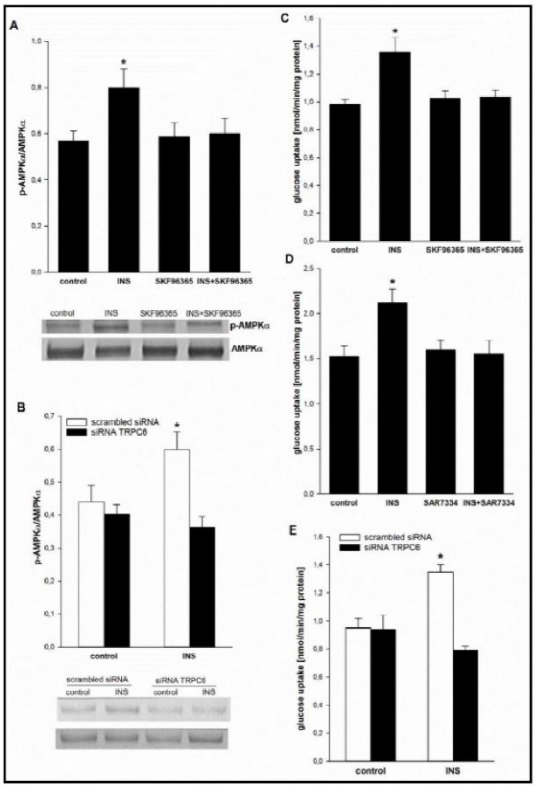

Fig. 3. TRPC6 channels mediate insulin-dependent activation of AMPK and glucose uptake in cultured rat podocytes. The effects of the TRPC6 inhibitor SKF96365 (A) and of downregulation of TRPC6 channels (B) on the insulin-dependent activation of AMPK. Cell lysates were subjected to immunoblotting analysis using anti-AMPKα and anti-p-AMPKα (Thr^{172}) antibodies. Values are reported as the means±SEMs of four independent experiments. *P<0.05 compared to control. The effects of the TRPC6 inhibitors SKF96365 (C), SAR7334 (D) and of downregulation of TRPC6 channels (E) on insulin-dependent increases in glucose uptake. Uptake measurements were performed after the addition of 1 μCi of [1,2-^{3}H]-deoxy-D-glucose diluted in non-radioactive glucose to a final concentration of 300 nM insulin. Values are reported as the means±SEMs of four to five independent experiments. *P<0.05 compared to control.

Insulin-mediated activation of downstream targets of Rho kinases is TRPC6-dependent

We next evaluated the influence of TRPC6 on the insulin-dependent regulation of downstream targets of Rho kinases. Insulin-stimulated activation of Rac1 activates the serine/threonine kinase PAK in skeletal muscle [22]. Activation of PAK involves phosphorylation of the Thr423 and Ser141 residues, dissociation of the dimer, and release of the catalytic domains [27]. In podocytes, insulin stimulation (300 nM, 5 min) increased PAK Ser141 phosphorylation by 71% (1.20±0.24 vs. control 0.70±0.03) and PAK Thr423 phosphorylation by 33% (1.54±0.11 vs. control 1.16±0.09), $P<0.05$ for both. Furthermore, we found that preincubating cells with SKF96365 (100 μM) or downregulation of TRPC6 by siRNA attenuated the effect of insulin on PAK phosphorylation in podocytes (Fig. 7). We also

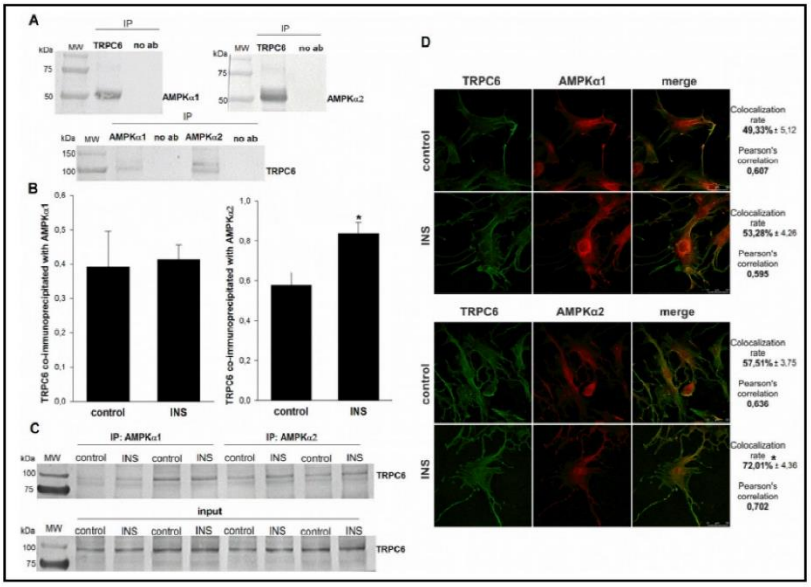

Fig. 4. The role of insulin in TRPC6 channel interactions with AMPKα subunits in cultured rat podocytes. (A) Immunoblots showing that the AMPKα1 and AMPKα2 subunits are associated with immunoprecipitated TRPC6 in podocyte extracts and that, conversely, TRPC6 is associated with immunoprecipitated AMPKα1 and AMPKα2. (B) Insulin increases the amount of TRPC6 that coimmunoprecipitates with AMPKα2. (C) Representative immunoblots. (D) Podocytes were seeded onto coverslips and incubated with or without insulin (300 nM, 5 min). The cells were than immunoblotted with anti-TRPC6 and anti-AMPKα1 or anti-AMPKα2 antibodies as indicated. Quantitative analysis of protein colocalization was performed with LAS AF 3.3.0 software (n=8–10, *P<0.05). The pixel intensities were quantified and the results are reported as Pearson's correlation coefficients and colocalization rates (%).

investigated the influence of TRPC6 activity on ROCK1 and ROCK2 levels in rat podocytes. ROCK is a major downstream effector of the RhoA kinase [28]. The TRPC6 inhibitor SKF96365 did not influence ROCK kinase levels (Fig. 8A, C). Moreover, downregulation of TRPC6 by siRNA decreased the ROCK1 level by 23% (from 0.644±0.036 to 0.488±0.014, $P<0.05$) without affecting ROCK2 expression (Fig. 8B, D). Taken together, these results suggest that downregulation of TRPC6 attenuated the insulin-dependent activation of downstream targets of Rho kinases in podocytes.

TRPC6 channels regulate the insulin-dependent remodeling of the actin cytoskeleton in podocytes

The dynamics of actin filament assembly/disassembly and its organization in cells are regulated by several actin-binding proteins, including ADF/cofilins [29]. In addition, PAK signals to cofilin in response to insulin, thereby facilitating cortical actin remodeling and glucose uptake in skeletal muscle cells [30]. Accordingly, next we examined the effects of insulin on cofilin phosphorylation in podocytes. Notably, cofilin is activated when it is dephosphorylated. We found that insulin stimulation (300 nM, 5 min) decreased the p-cofilin level by about 25% (1.46±0.11 vs. control 1.95±0.10, $P<0.05$, Fig. 9) in cultured rat podocytes. Preincubation of the cells with SKF96365 (Fig. 9A) or downregulation of TRPC6 by siRNA (Fig. 9B) abolished the effect of insulin on cofilin phosphorylation.

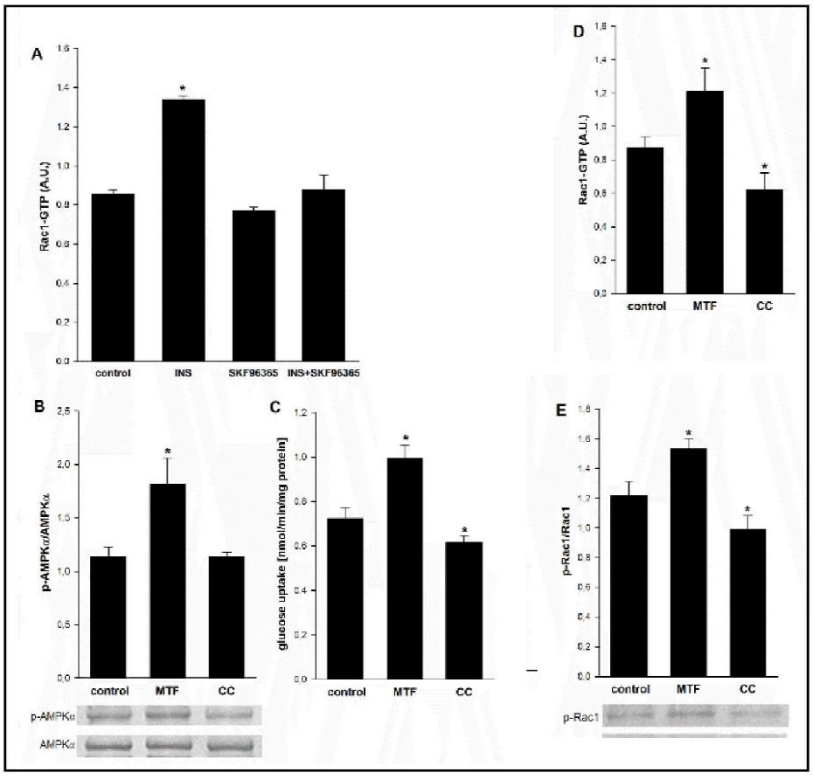

Fig. 5. The influence of TRPC channels and AMPK on Rac1 activity in cultured rat podocytes. (A) Cells were incubated for 5 min with 300 nM insulin in the presence or absence of the TRPC6 inhibitor SKF96365 (10 μM, 20 min). Cell lysates (20 μg) were subjected to the G-LISA assay, and absorbance was read at 490 nm. The data are background-subtracted. Values are reported as the means±SEMs of six independent experiments. *P<0.05 compared to control. The effect of the AMPK activator metformin (2 mM) and the AMPK inhibitor compound C (100 μM) on AMPK activity (B) and on glucose uptake (C). The effect of AMPK on the regulation of Rac1 activity (D) and on serine 71 phosphorylation of Rac1 (E). Values are reported as the means±SEMs of four to six independent experiments. *P<0.05 compared to control.

Phosphorylation of cofilin on serine3 leads to reduced actin binding and to actin depolymerization [31]. We found that insulin increased the colocalization of cofilin with actin and that TRPC6 inhibition decreased this effect in podocytes (Fig. 9C). Taken together, these results support accumulating evidence that TRPC6 participates in the regulation of actin dynamics in podocytes and suggest that this function is mediated in part by cofilin.

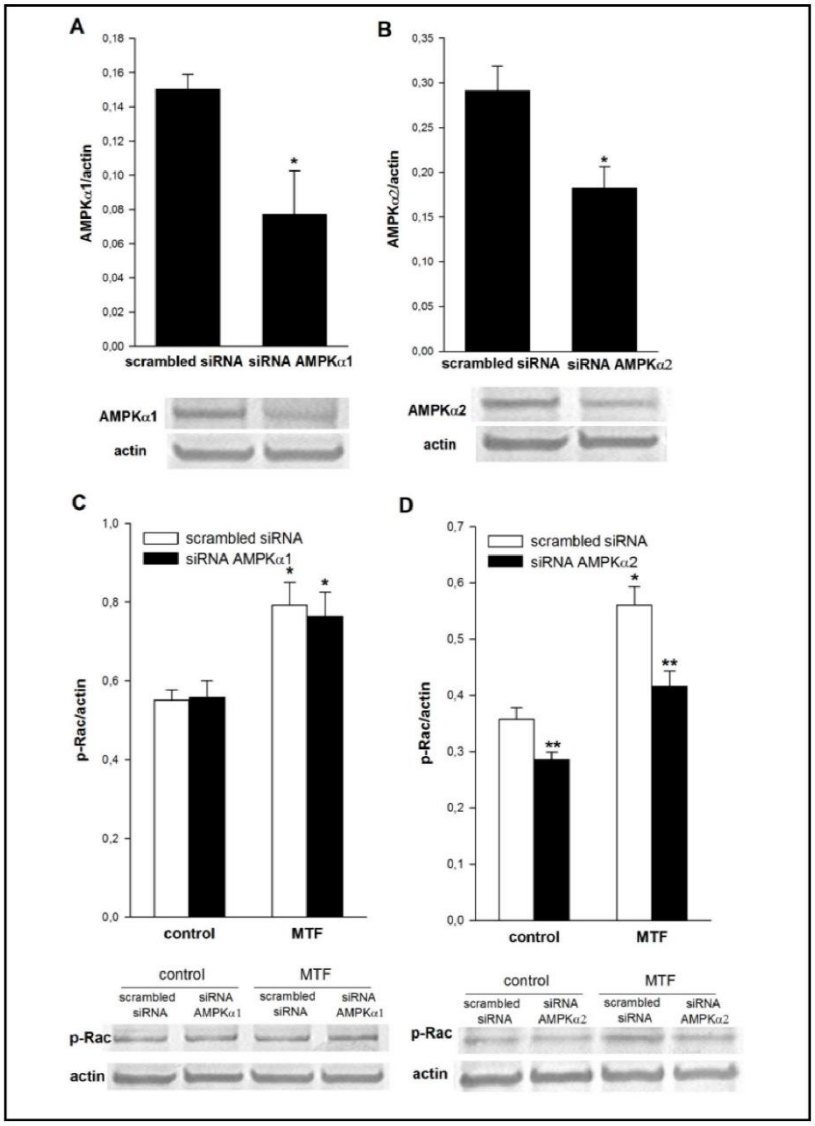

Fig. 6. The role of AMPKα1 and AMPKα2 subunits in the regulation of Rac1 activity. The effects of small interfering RNA targeting transcripts of the AMPKα1 or AMPKα2 subunit in cultured rat podocytes. Densitometry was performed to evaluate the expression of AMPKα1 (A) and AMPKα2 (B), and the band signals were normalized using signals of actin bands. Controls were transfected with scrambled siRNA. The influence of downregulation of AMPKα1 (C) and AMPKα2 (D) on Rac1 phosphorylation. Values are reported as the means±SEMs of four independent experiments. *P<0.05 compared to control, **P<0.05 compared to the appropriate control with scrambled siRNA.

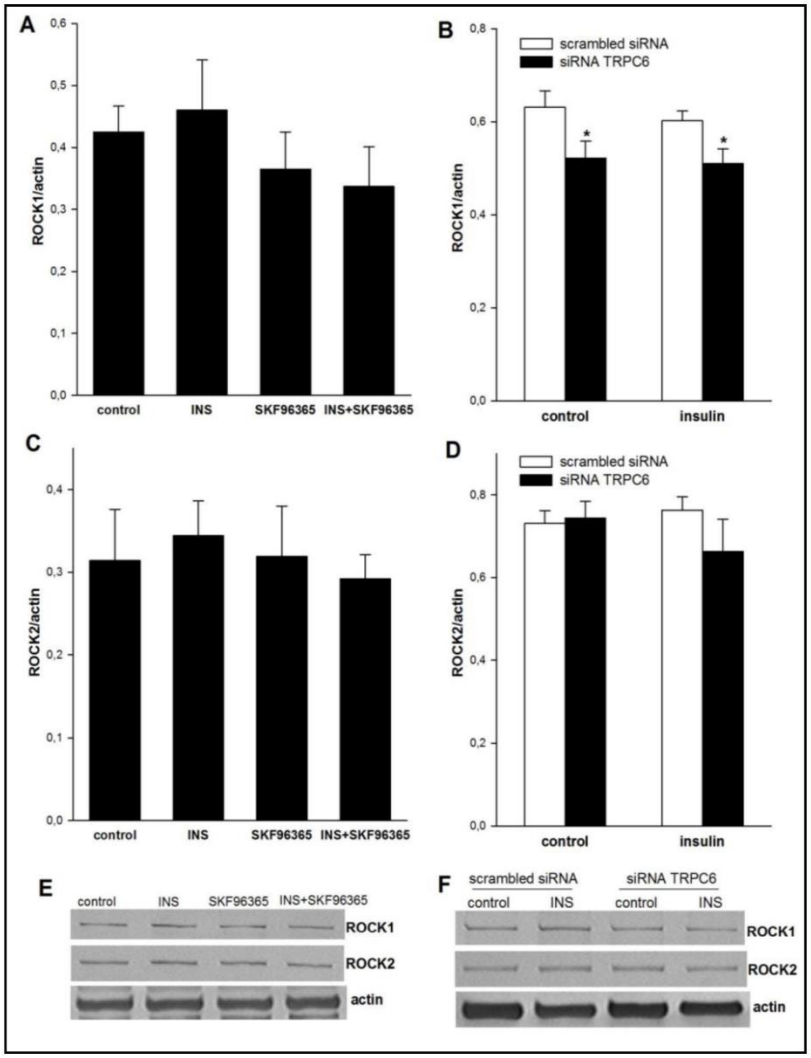

Fig. 8. The influence of TRPC6 channels on ROCK1 and ROCK2 expression in podocytes. Podocytes were incubated for 5 min with 300 nM insulin in the presence or absence of the TRPC channel inhibitor SKF96365 (A, C) or TRPC6 siRNA (B, D). Densitometric analysis of the corresponding bands was performed, and values are reported as the ratios of the band intensities for ROCK1 or ROCK2 to actin. Values are reported as the means±SEMs of four to six independent experiments. *P<0.05 compared to control.

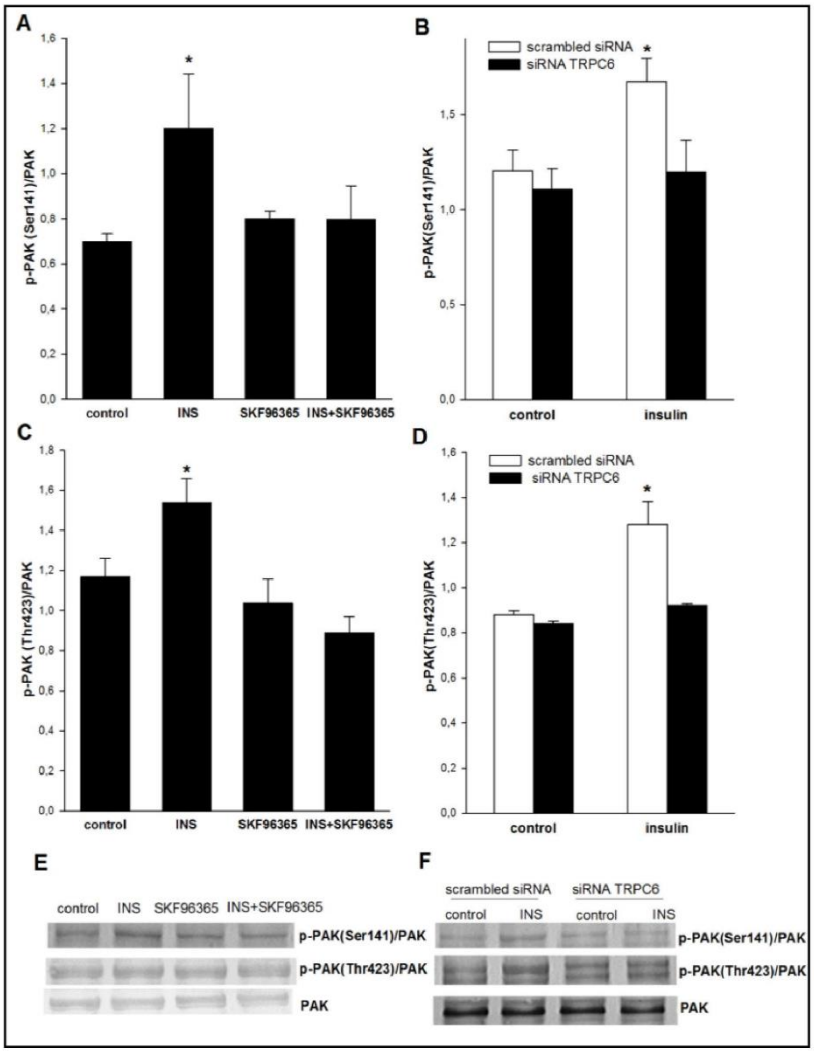

Fig. 7. The role of TRPC channels in insulin-mediated phosphorylation of p21-activated kinase (PAK) in cultured rat podocytes. Podocytes were incubated for 5 min with 300 nM insulin in the presence or absence of the TRPC channel inhibitor SKF96365 (A, C) or TRPC6 siRNA (B, D). Densitometric analysis of the corresponding bands was performed, and values are reported as the ratios of the band intensities for p-PAK (Ser^{141}) or p-PAK (Thr^{423}) to PAK. Values are reported as the means±SEMs of four to six independent experiments. *P<0.05 compared to control.

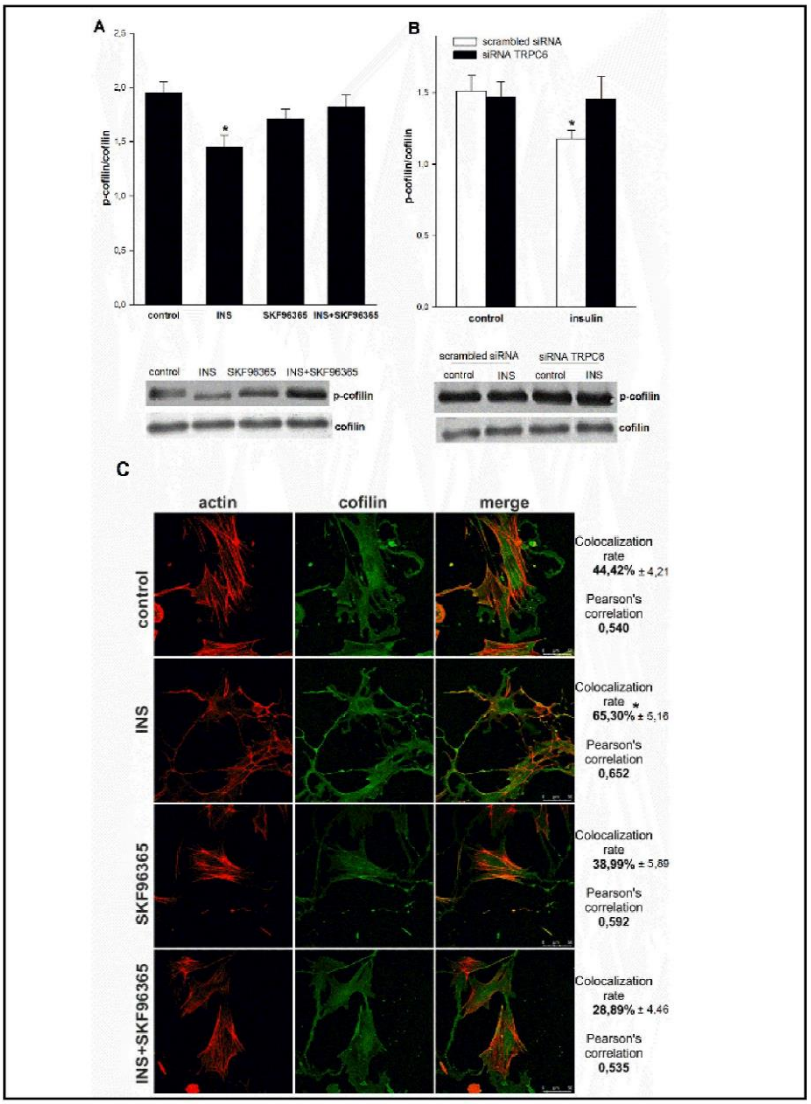

Fig. 9. TRPC6 channel-mediated insulin-dependent activation of cofilin and regulate insulin-induced changes in the colocalization of actin and cofilin in cultured rat podocytes. The effects of the TRPC channel inhibitor SKF96365 (A) or TRPC6 siRNA (B) on the insulin-dependent dephosphorylation of cofilin. Values are reported as the means±SEMs of four to six independent experiments. *P<0.05 compared to untreated podocytes. (C) Rat podocytes seeded onto coverslips were incubated for 5 min with 300 nM insulin in the presence or absence of the TRPC channel inhibitor SKF96365. The cells were then immunostained with anti-cofilin antibody and isothiocyanate phalloidin to detect actin. Quantitative analysis of protein colocalization was performed with LAS AF 3.3.0 software (n=10, *P<0.05). The pixel intensities were quantified, and the results are reported as Pearson's correlation coefficients and colocalization rates (%).

Discussion

This study showed that the TRPC6-dependent activation of AMPKα2 signaling pathways is a novel mechanism for the insulin-mediated regulation of actin cytoskeleton dynamics and glucose uptake in podocytes. The proposed mechanism, which is based on our data, is shown in Fig. 10. First, insulin-stimulated glucose uptake and actin cytoskeleton reorganization depends on TRPC6 channel activation. Second, activation of the AMPKα signaling pathway is TRPC6-dependent. Third, insulin regulates the interaction of TRPC6 with AMPKα2 in cultured rat podocytes. Fourth, AMPK and TRPC6 activation are required to stimulate Rac1 signaling pathways.

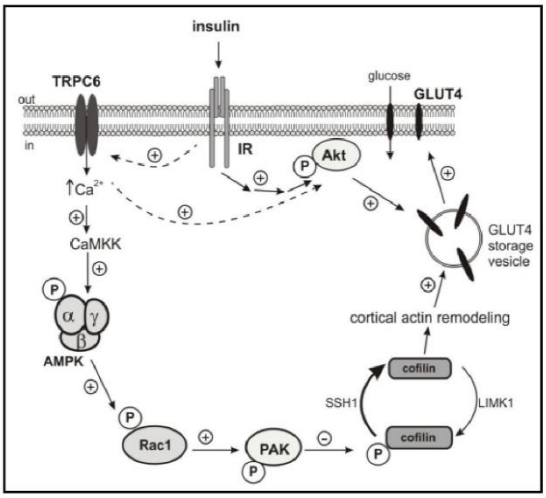

Fig. 10. The proposed mechanism of insulin involvement in TRPC6-AMPK pathways that are involved in the cytoskeleton regulation and in the regulation of glucose uptake in podocytes.

We recently demonstrated that the insulin-mediated regulation of the contractile apparatus and filtration barrier permeability relies on TRPC6-dependent activation of PKGIα signaling pathways. Our studies found that insulin induced the reorganization of actin via TRPC6-dependent Ca^{2+} entry [11]. The present study confirmed the role of TRPC6 in the regulation of insulin-dependent signaling pathways and actin dynamics. Moreover, we demonstrated for the first time that TRPC6 plays a role in the regulation of AMPK activity and glucose uptake in cultured rat podocytes.

AMPK is a major regulator of insulin-dependent glucose uptake and insulin signaling in podocytes [18]. Phosphorylation of AMPK at Thr172 is required for the activation of AMPK [32], and Ca^{2+}/calmodulin-dependent CaMKK-β activates AMPK in various cell types, including podocytes [33-35]. CaMKK-β activity depends on increases in intracellular calcium, and insulin induces calcium flux into cells [9, 11]. Here we demonstrated that insulin induced a change in AMPK phosphorylation through TRPC6 activation. Our results showed that insulin effect on glucose uptake in podocytes depends on AMPK activity which is regulated by TRPC6. Thus, we conclude that TRPC6 is upstream regulator of AMPK activity in podocytes. We also found, that insulin increased the amount of TRPC6 colocalized with the AMPKα2 subunit in podocytes. Others have also demonstrated a link between TRPC and AMPK activity. For example, TRPC1 knockdown in endothelial cells prevents PAR1 agonist peptide-induced AMPKα phosphorylation [36]. It is possible that AMPK and TRPC3 are part of the same signaling pathway that affects the cytoskeletal network and erythrocyte survival [37]. We assume that AMPK function depends on TRPC6-regulated calcium ion flux and we demonstrated that inhibiting TRPC6 channels and knocking down TRPC6 expression blocked insulin-dependent glucose uptake. We observed that the basal glucose uptake was not different between SKF96365- or SAR7334-treated and TRPC6 siRNA-treated cells versus control cells; this is consistent with the fact that basal glucose uptake does not involve GLUT4 translocation.

Conclusion

In summary, our results add to accumulating evidence that TRPC6 participates in the insulin-mediated regulation of actin dynamics in podocytes and suggest that this function is mediated, at least in part, by cofilin. Thus, cofilin dephosphorylation might be attractive as a pharmacological target in order to ensure appropriate actin turnover in diabetes with insulin resistance and to ensure GLUT4 function and glucose uptake.

Acknowledgements

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Disclosure Statement

None of the authors have any competing interests.

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Recently, it was demonstrated that SAR7334 is particularly useful as it blocks TRPC6 at low nanomolar levels (IC_{50} 9.5 nM), blocks TRPC3 and TRPC7 at orders of magnitude higher concentrations (IC_{50} 282 and 226 nM, respectively) [38]. Additionally, Dryer's group showed that SAR7334 is selective inhibitor of TRPC6 in podocytes in low nanomolar concentration [39]. The higher concentration (1 μM) cannot entirely exclude a role for TRPC3-dependent entry of Ca^{2+} into podocytes.

Insulin and muscle contraction are the major known mediators of GLUT4 translocation under physiological conditions [40, 41]. The importance of podocyte insulin signaling in the pathogenesis of diabetic kidney disease is suggested by the observation that podocytes isolated from diabetic db/db mice cannot phosphorylate Akt in response to insulin and do not translocate GLUT4 to the plasma membrane after insulin stimulation [42]. We suggest that insulin induces the TRPC6-dependent entry of Ca^{2+} into podocytes, the translocation of GLUT4 to the membrane, and, consequently, an increase in glucose uptake in podocytes. However, this needs further investigation. Others have shown that TRPC3 knockdown reduces insulin-mediated glucose uptake in skeletal muscle cells. Moreover, TRPC3 and GLUT4 colocalize in the t-tubule system, which is responsible for insulin-dependent glucose uptake [43]. It is clear that the ability of podocytes to precisely regulate intracellular Ca^{2+} levels plays a crucial role in proper glucose uptake.

The rho family GTPases act as molecular switches that are best known for their pivotal roles in the dynamic regulation of the actin cytoskeleton. The mammalian Rho family has at least 20 distinct members, of which RhoA, Rac1, and Cdc42 are the most extensively studied [44]. Rac1 is a major regulator of actin remodeling, and cytoskeletal rearrangement is required for GLUT4 translocation in response to insulin [26].

These findings suggest that Rac1 and downstream signaling to the actin cytoskeleton constitute an important dysfunctional pathway in insulin-resistant states. In podocytes, cells that are sensitive to insulin, this mechanism may not be examined. The results presented here provide evidence that activation of AMPKα2 and TRPC6 are required for insulin-dependent Rac1 signaling pathway stimulation in podocytes. We further demonstrated that the insulin-mediated activation of downstream targets of Rho kinases was TRPC6 dependent. Inhibition of TRPC6 activity attenuated the effect of insulin on PAK phosphorylation, decreasing ROCK1 expression and cofilin activation. Insulin-stimulated Rac1 also activates the PAK1 serine/threonine kinase in skeletal muscle [22]. Moreover, PAK1 inhibition reduces insulin-induced cortical actin remodeling, cofilin activation, and GLUT4 translocation [30]. It is likely that the diminished activity of Rac1 and PAK1 in an insulin-resistant state leads to functional defects in proteins involved in actin remodeling that are necessary for glucose uptake in podocytes. Another group also demonstrated that the ability of insulin to increase glucose transport is attenuated by ROCK1 suppression [45]. This suggests that a decrease in ROCK1 expression in podocytes after downregulation of TRPC6 additionally reduces insulin-dependent glucose transport.

Rac1 is also necessary for the assembly of the membrane-located superoxide-producing NADPH oxidase (NOX) complexes, where it is required for the electron transfer from NADPH to oxygen. NOX isoforms NOX1 and NOX2 are activated via Rac1 having essential roles in physiology and in several human diseases [46]. Recently it was demonstrated that NADPH oxidase is also required to TRPC6 activation in the presence of insulin [11].

Taken together, our data demonstrate that insulin induces the activation of PAK1 and cofilin-1, increases the colocalization of cofilin with actin, and, as a consequence, induces actin remodeling and glucose uptake. Cofilin disassembles actin, thereby maintaining the flexibility of actin remodeling and supporting the regeneration of free monomeric actin for further polymerization. This active cycling of actin as mediated by insulin is necessary for actin dynamics and to facilitate GLUT4 translocation to the surface of muscle cells [23].

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The PKGIα/VASP pathway is involved in insulin- and high glucose-dependent regulation of albumin permeability in cultured rat podocytes

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Podocytes, the principal component of the glomerular filtration barrier, regulate glomerular permeability to albumin via their contractile properties. Both insulin- and high glucose (HG)-dependent activation of protein kinase G type Iα (PKGIα) cause reorganization of the actin cytoskeleton and podocyte disruption. Vasodilator-stimulated phosphoprotein (VASP) is a substrate for PKGIα and involved in the regulation of actin cytoskeleton dynamics. We investigated the role of the PKGIα/VASP pathway in the regulation of podocyte permeability to albumin. We evaluated changes in high insulin- and/or HG-induced transepithelial albumin flux in cultured rat podocyte monolayers. Expression of PKGIα and downstream proteins was confirmed by western blot and immunofluorescence. We demonstrate that insulin and HG induce changes in the podocyte contractile apparatus via PKGIα-dependent regulation of the VASP phosphorylation state, increase VASP colocalization with PKGIα, and alter the subcellular localization of these proteins in podocytes. Moreover, VASP was implicated in the insulin- and HG-dependent dynamic remodelling of the actin cytoskeleton and, consequently, increased podocyte permeability to albumin under hyperinsulinaemic and hyperglycaemic conditions. These results indicate that insulin- and HG-dependent regulation of albumin permeability is mediated by the PKGIα/VASP pathway in cultured rat podocytes. This molecular mechanism may explain podocytopathy and albuminuria in diabetes.

Keywords: high glucose; insulin; podocytes; protein kinase G type Iα;VASP.

Abbreviations: DN, diabetic nephropathy; FPs, foot processes; HG, high glucose; MLC, myosin light chain; PKGIα, protein kinase G type Iα; pMLC, MLC phosphorylation; pVASP Ser239, VASP phosphorylation at Ser239; SD, slit diaphragm; SFM, serum-free medium; SG, standard glucose; siRNAs, small-interfering RNAs; VSMCs, vascular smooth muscle cells; VASP, vasodilator-stimulated phosphoprotein; VEGF, vascular endothelial growth factor.

Chronic hyperglycaemia and hyperinsulinemia are conducive to the development of diabetic nephropathy (DN), one of the main complications of diabetes mellitus (*1*). The first clinical sign of DN is albuminuria due to increasing glomerular barrier permeability (*2*). The main component of the glomerular filtration barrier is podocytes, which are highly specialized, terminally differentiated cells that surround glomerular capillaries (*3*). Podocytes consist of a cell body, major processes and interdigitating foot processes (FPs) forming the filtration slit. Neighbouring FPs are connected by cell–cell junctions called a slit diaphragm (SD), which is the major glomerular filter sieve impermeable to plasma proteins, such as albumin (*2*, *4*, *5*).

Podocytes play a key role in regulating glomerular filtration barrier permeability through actin filament reorganization in the FPs. The FPs are actin-rich structures in which longitudinally orientated thick actin bundles are surrounded by a sub-plasmalemmal network of cortical branched actin filaments that link the SD domain, the basal domain of FPs, and the apical domain of FPs to the actin bundle (*6*). The FP actin cytoskeleton is a complex contractile apparatus that enables podocytes to be dynamic and rearrange themselves rapidly depending on the needed changes in filtration; therefore, podocyte morphology and function are strictly associated with the actin cytoskeleton (*7*). Podocyte injury is characterized by alterations in SD function, leading to the effacement of FPs, increased podocyte motility and increasing proteinuria (*8*, *9*). In patients suffering from diabetes-associated nephropathy, podocyte structure and function are altered and correlate with the urinary albumin excretion rate (*10*, *11*).

Insulin is a hormone that directly influences podocyte actin rearrangement and is extremely important in maintaining glomerular filtration barrier integrity. Stimulation of the insulin receptor causes retraction of actin-rich FPs, increasing cell motility and migration, whereas knocking down insulin receptor in mice podocytes leads to FP decline, resulting in albuminuria (*12*). *In vitro* studies have confirmed a stimulating effect of insulin on podocyte albumin permeability (*13*, *14*). The evidence indicates a significant relationship between insulin signalling, actin cytoskeleton reorganization and glomerular permeability.

Protein kinase G type Iα (PKGIα) is involved in insulin-dependent regulation of the glomerular filtration barrier (*14–16*). The PKGIα isoform present in cultured rat podocytes is activated by insulin through the formation of interprotein disulphide bonds between its two subunits (*17*). Piwkowska *et al.* (*14*) demonstrated that insulin influences actin rearrangement and increases podocyte permeability to albumin in a PKGIα-dependent manner. Moreover, a high glucose (HG) concentration also augments podocyte permeability to albumin in a PKGIα-dependent manner, suggesting a crucial role of PKGIα in the development of DN (*18*, *19*).

Contractile system of cells depends on myosin light chain (MLC) phosphorylation at Ser19 (*20*). The phosphorylation state of MLC may serve as a biological marker of PKGIα activation in cultured rat podocytes. Insulin- and HG-induced activation of PKGIα decreases the level of MLC phosphorylation (pMLC; *18*, *21*). This mechanism leads to relaxation of the podocytes and increases their permeability to albumin (*14*, *17*).

PKGIα activation, actin reorganization, and alterations in protein permeability in a podocyte filtration monolayer are closely related. Unfortunately, the indirect link between PKGIα activation and actin filament reorganization in FPs is still not known, and further studies are needed to explain the effect of their mutual interaction on glomerular barrier permeability.

Vasodilator-stimulated phosphoprotein (VASP) belongs to the group of proteins that regulate the organization of the actin cytoskeleton. VASP belongs to the family of Ena/VASP actin-associated proteins (*22*) and localizes to the actin microfilaments, focal adhesions, stress fibres, cell–cell junctions and dynamic membrane regions (*23–26*). Functionally, VASP participates in actin filament elongation and cell migration, whereas phosphorylation of VASP influences its subcellular localization, affects the regulation of actin polymerization and attenuates VASP affinity for actin by 40-fold (*27–29*). PKG phosphorylates VASP at Ser239 (*30*), the functional consequence of which is inhibition of actin filament assembly (*27*, *31*). In vascular smooth muscle cells (VSMCs), effective vascular contractility depends on the elongation of actin filaments, which is mediated by VASP (*29*). Activation of the PKG/VASP signalling pathway, including VASP phosphorylation at Ser239 (pVASP Ser239), results in vascular relaxation in VMSCs (*32*). Unfortunately, little is known about the role of VASP and its phosphorylated form in podocyte function. Recently, inhibition of PAR1 phosphatase was demonstrated to result in augmented podocyte motility, dependent on pVASP Ser239 (*33*). These data suggest that the properties of VASP render it a suitable receptor-mediated transmitter of molecular signals to the podocyte actin cytoskeleton, and that it may be involved in podocyte pathophysiology.

In this study, we investigated whether VASP is involved in regulating the podocyte actin cytoskeleton and permeability through insulin- and/or HG-dependent activation of PKGIα. This research will provide new insight into the role of VASP in podocyte function under physiological and pathophysiological conditions. Moreover, our research suggests VASP phosphorylation state as a potential biomarker of DN.

Materials and Methods

Preparation and culture of rat podocytes

All experiments were performed in accordance with directive 2010/63/EU for animal experiments and the protocol approved by the local ethics committee of UTP University of Science and Technology, Bydgoszcz, Poland.

Female Wistar rats weighing 120–140 g were used for primary podocyte culture as described previously (*14*). All experiments were performed using podocytes cultured for 12–20 days. Cell phenotypes were confirmed by podocyte-specific antibodies against Wilms tumor-1 protein (Biotrend Koeln, Germany) and synaptopodin (Progen, Heidelberg, Germany). Previous studies had showed that cells incubated for 5 days in HG become insulin resistant (*34*). Therefore, before different experiments, podocytes were incubated for 5 days in SG medium (RPMI-1640 medium, 10% fetal bovine serum (FBS), 11.1 mM D-glucose) or HG medium (RPMI-1640 medium, 10% FBS, 30 mM D-glucose) and in the presence or absence of PKGIα modulators. Medium with appropriate modulator was changed two times. L-glucose served as an osmotic control for HG.

Western blotting

To obtain podocyte 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 protease (Sigma-Aldrich) and phosphatase (Roche) inhibitor cocktails and homogenized at 4°C by scraping. Proteins in the supernatant (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-VASP (1:28000, Sigma-Aldrich), anti-p-VASP (Ser239) (1:667, Abcam), anti-cGKIα (1:400, Santa Cruz Biotechnology), anti-p-MLC2 (Ser19) (1:667, Cell Signaling Technology) and anti-actin (1:16000, Sigma-Aldrich). To detect the primary antibodies, the membranes were incubated with the appropriate alkaline phosphatase-labelled secondary antibodies (Sigma-Aldrich). The protein bands were visualized using the colorimetric 5-bromo-4-chloro-3-indolyl-phasphate/nitroblue tetrazolium system. Densitometric quantification of bands was performed using Quantity One software (Bio-Rad).

Small-interfering RNA transfection

Podocytes were transfected with small-interfering RNAs (siRNAs) targeting VASP, PKGIα or non-silencing siRNA (scrambled siRNA, negative control; OriGene). Cells were cultured in RPMI-1640 medium 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 siRNAs using siRNA Transfection Reagent (OriGene) according to the manufacturer's instructions. Briefly, the targeted siRNA or scrambled siRNA was diluted in Transfection Medium (final concentration, 80 nM), mixed with siRNA Transfection Reagent, and incubated for 30 min at room temperature. Next, the Transfection Medium was added to the transfection mixture, mixed gently, and added to the podocytes. After 7 h, growth medium supplemented with 2× higher concentrations of FBS and antibiotics was added to the cells. Afterwards, the podocytes were incubated for an

additional 24 h. After transfection, gene silencing was checked at the protein level by western blotting.

The siRNA targeting VASP was synthesized by OriGene (cat. no. SR515513). A set of VASP Rat siRNA Oligo Duplex consists of three unique 27mer siRNA duplexes. The experiments with all these three siRNA Duplexes (A–C) were conducted. The siRNA targeting PKGIα was synthesized by Santa Cruz Biotechnology (cat. no. sc-270330) and consisted of pools of three to five target-specific 19 × 25 nucleotide siRNAs.

Immunofluorescence

Podocytes were seeded on coverslips coated with Type 1 collagen (Becton Dickinson Labware, Becton, UK) and cultured in RPMI-1640 medium supplemented with 10% FBS. Cells were fixed in PBS plus 4% formaldehyde for 20 min at room temperature. Fixed podocytes were permeabilized with 0.1% Triton-X for 3 min and then blocked with PBSB solution [phosphate-buffered saline (PBS) plus 2% FBS, 2% bovine serum albumin (BSA) and 0.2% fish gelatin] for 1 h. After blocking, cells were incubated with anti-VASP (1:30) and anti-PKGIα (1:15) antibodies in PBSB at 4°C for 1.5 h. The primary antibodies 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 (1:750) or Alexa Fluor 546 (1:750). Actin was stained using Alexa Fluor 633 phalloidin (1:200). Specimens were imaged using a confocal laser scanning microscope (Leica SP8X) with a 63× oil immersion lens.

Permeability assay

Transepithelial permeability to albumin was investigated by measuring the diffusion of fluorescein isothiocyanate (FITC)-labelled BSA (Sigma-Aldrich) across the podocyte monolayer as described previously by Oshima *et al.* (*35*, *36*), with minor modifications. Briefly, podocytes (25×10^3 cells/well) were seeded on 3-μm membrane pore size cell culture inserts coated with type IV collagen (Corning) and placed in 24-well plates. Transwell permeability experiments were conducted on differentiated cells between 7 and 15 days post-seeding. Before the experiments, podocytes were washed twice with PBS and medium on both sides of the insert and the medium replaced with serum-free RPMI-1640 medium (SFM). After 2 h, the medium in the upper compartment was replaced with fresh SFM supplemented with 1 mg/ml FITC-albumin. After 1 h incubation, the solution from the upper chamber was transferred to a 96-well plate and the absorbance of FITC-albumin measured at 490 nm using an EL808 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis

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

Results

Short-term incubation with insulin increases PKGIα-dependent VASP phosphorylation in cultured rat podocytes

Insulin-mediated PKGIα activation greatly affects podocyte functioning (*15*, *37*, *38*). Therefore, we questioned whether insulin has an impact on pVASP Ser239. The expression of total VASP did not change significantly after administration of insulin or 8-Br-cGMP (Fig. 1A), but we found that podocytes treated with insulin (300 nM, 5 min) or PKG activator 8-Br-cGMP (100 μM, 5 min) had significantly elevated basal levels of phosphorylated VASP Ser239, by 23% (0.86 ± 0.06 versus 0.69 ± 0.04, $n = 9$, $P < 0.05$, Fig. 1B) and 174% (1.89 ± 0.24 versus 0.69 ± 0.04, $n = 9$, $P < 0.05$, Fig. 1B), respectively. Pre-incubation of podocytes with PKG inhibitor Rp-8-Br-cGMPS abolished the effect of insulin on VASP phosphorylation (0.57 ± 0.04 versus 0.86 ± 0.06, $n = 8$–9, $P < 0.05$, Fig. 1B). Moreover, immunofluorescent staining of podocytes to visualize localization of PKGIα and VASP confirmed that both insulin and 8-Br-cGMP increased the interaction of these two

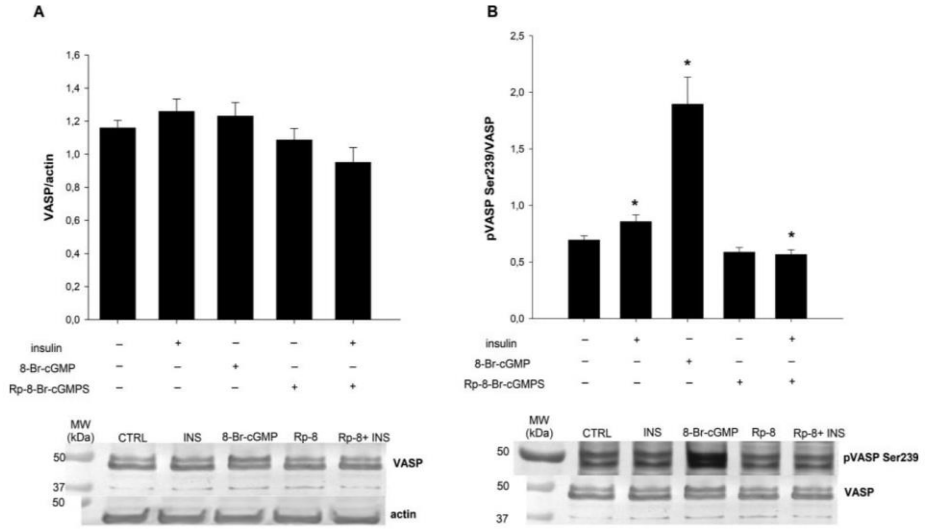

Fig. 1. The effects of PKG modulators on VASP expression and its phosphorylation level in podocytes. Podocytes were treated with insulin (INS; 300 nM, 5 min), PKG activator 8-Br-cGMP (100 μM, 5 min), and/or PKG inhibitor Rp-8-Br-cGMPS (Rp-8; 100 μM, 20 min pre-incubation). Densitometric analysis of the corresponding bands was performed, and values are presented as the ratio of band intensity for VASP to actin (**A**) and pVASP Ser239 to VASP (**B**). Values are the mean ± SEM, $n = 9$–10, $^*P < 0.05$.

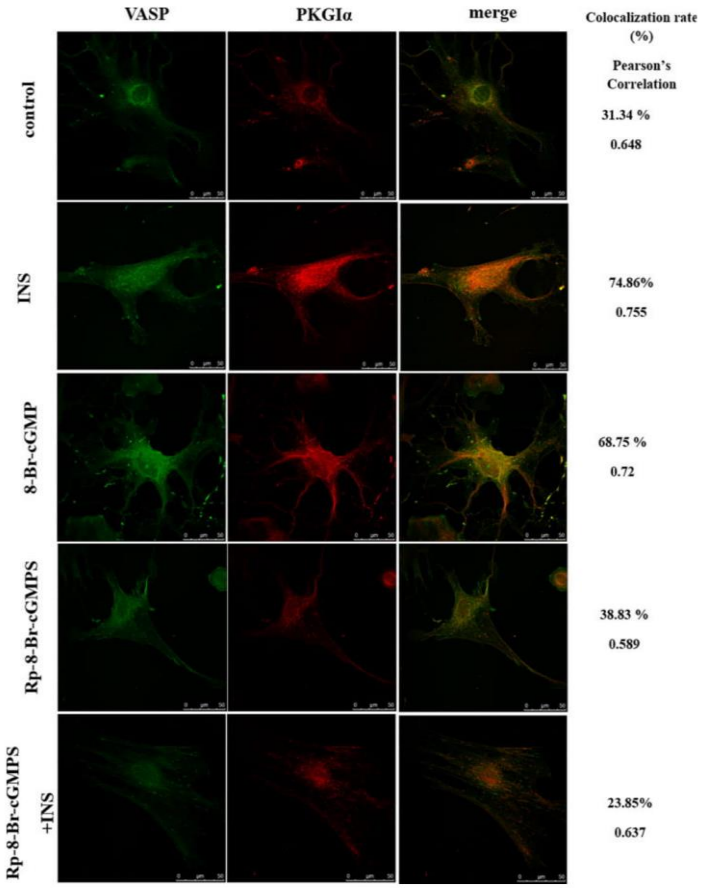

Fig. 2. Subcellular distribution of PKGIα and VASP in cultured rat podocytes. The merged images show colocalization of PKGIα with VASP in differentiated rat podocytes. Cells seeded onto coverslips were incubated with insulin (INS, 300 nM, 5 min), 8-Br-cGMP (100 μM, 5 min), and/or Rp-8-Br-cGMPS (100 μM, pre-incubation 20 min). Podocytes were immunostained with anti-PKGIα (1:15; Santa Cruz Biotechnology) and anti-VASP (1:30; Sigma Aldrich) antibodies. Quantitative analysis of protein colocalization was performed with LAS AF 3.3.0 software ($n = 4$–5). The pixel intensities were quantified and the results presented as Pearson's correlation coefficients and colocalization rates (%).

proteins and changed their localization from perinuclear to a more even distribution in the cell (Fig. 2). These results suggest that PKGIα activation is required for insulin-induced pVASP Ser239, and phosphorylation at this site alters PKGIα and VASP localization in podocytes.

Long-term incubation with insulin and HG increase VASP expression in cultured rat podocytes

Insulin and HG mediate PKGIα activation in podocytes (*18*). We hypothesized that VASP expression is augmented under hyperinsulinaemic and hyperglycaemic conditions. Podocytes were cultured in standard glucose (SG, 11 mM) or HG (30 mM) medium in the presence or absence of insulin (300 nM) for 5 days. Insulin and HG increased the level of total VASP protein by 35% (0.83 ± 0.03 to 1.12 ± 0.08, $n = 4$, $P < 0.05$) and 29% (0.83 ± 0.03 to 1.07 ± 0.07, $n = 4$, $P < 0.05$), respectively (Fig. 3A). Immunofluorescence experiments confirmed the increased PKGIα and VASP colocalization in podocytes cultured under hyperinsulinaemic conditions (from 25.78% to 38.65%, $n = 3$, $P < 0.05$, Fig. 3B). In addition, long-term incubation of podocytes with insulin led to an enhancement of the immunofluorescent signal and perinuclear localization of PKGIα and VASP (Fig. 3B). Under hyperglycaemic conditions, colocalization of PKGIα and VASP significantly increased, correlating with a more even distribution of these proteins in podocytes compared with SG (Fig. 3B).

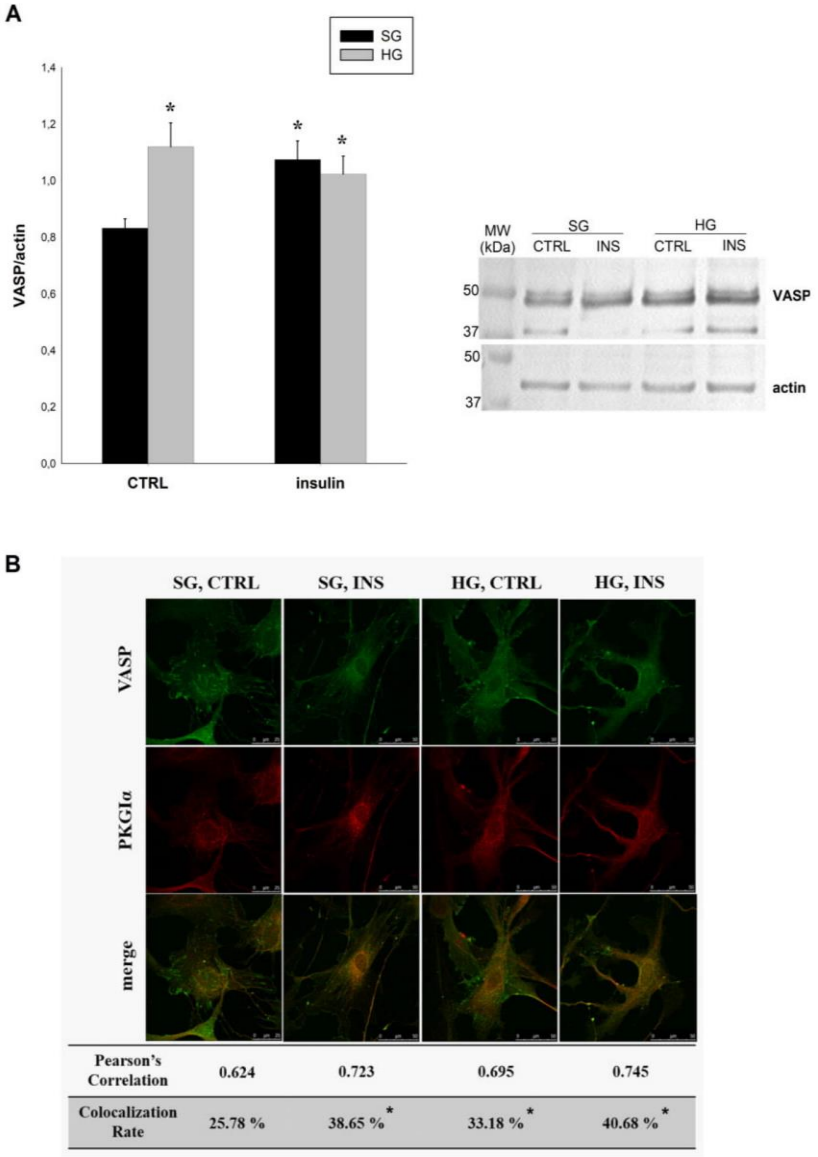

Fig. 3. The influence of insulin and HG on VASP expression in rat podocytes. Insulin (INS, 300 nM) increased VASP levels in podocytes exposed to SG (11 mM) or HG (30 mM) (**A**). Colocalization of PKGI and VASP in podocytes cultured in SG or HG medium in the presence or absence of insulin (300 nM, 5 days) (**B**). Densitometric analysis was performed to establish the amount of VASP protein under the different conditions. Actin was used as a loading control. Quantitative analysis of protein colocalization was performed using LAS AF 3.3.0 software. The pixel intensities were quantified and evaluated by Pearson's correlation to derive the colocalization rate (%). Values are the mean ± SEM, $n = 3$–4, $^*P < 0.05$ compared with SG.

To confirm that PKGIα interacted with VASP under SG and HG conditions, podocytes were treated with Rp-8-Br-cGMPS (50 μM), a PKG inhibitor, for 5 days. Rp-8-Br-cGMPS decreased the amount of VASP protein by 32% (0.72 ± 0.05 to 0.49 ± 0.02, $n = 3$, $P < 0.05$) and 48% (0.87 ± 0.03 to 0.45 ± 0.06,

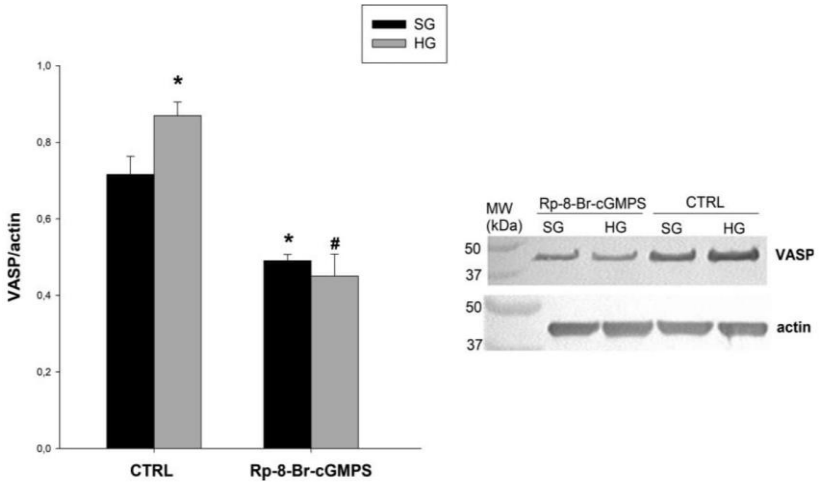

Fig. 4. The effect of PKG inhibitor on VASP expression in rat podocytes. Cells were cultured in SG (11 mM) or HG (30 mM) medium for 5 days in the presence or absence of 50 μM Rp-8-Br-cGMPS, a PKG inhibitor. Values are mean ± SEM, $n = 3$, $^{*}P < 0.05$ compared do SG, $^{\#}P < 0.05$ compared with HG.

$n = 3$, $P < 0.05$) in podocytes treated with SG and HG, respectively (Fig. 4).

These findings suggest that activation of PKGIα may mediate enhanced VASP expression and alter VASP functioning in podocytes by affecting its phosphorylation and localization under hyperinsulinaemic and hyperglycaemic conditions.

Downregulation of PKGIα expression in podocytes disturbs VASP phosphorylation at Ser239

Phosphorylation at Ser239 of VASP is a useful marker for monitoring both cGMP-dependent and cGMP-independent activation of PKG (*39*). To determine whether increased VASP Ser239 phosphorylation is a consequence of PKGIα activation, we transfected podocytes with PKGIα siRNA, reducing the quantity of this protein by 40% (0.76 ± 0.04 versus 0.46 ± 0.04, $n = 9$, $P < 0.05$) under SG conditions and 48% (0.88 ± 0.06 versus 0.46 ± 0.02, $n = 9$, $P < 0.05$) under HG conditions (Fig. 5A). In total, 49.5% transfection efficiency was obtained with the PKGIα siRNA (Fig. 6B).

Our results show that PKGIα regulates the VASP phosphorylation state. PKGIα gene silencing decreased the basal level of pVASP Ser239 by 47% (0.93 ± 0.02 versus 0.43 ± 0.06, $n = 4$, $P < 0.05$; Fig. 5B) and the basal level of VASP by 10% (1.37 ± 0.03 versus 1.24 ± 0.03, $n = 5$, $P < 0.05$, Fig. 5C). Rp-8-Br-cGMPS treatment also significantly reduced the quantity of pVASP Ser239 (Fig. 5B).

The data also indicate that HG-dependent activation of PKGIα results in increased pVASP Ser239. This effect was abolished by both PKGIα gene silencing (0.88 ± 0.06 versus 0.46 ± 0.02, $n = 9$, $P < 0.05$) and Rp-8-Br-cGMPS (1.09 ± 0.06 versus 0.77 ± 0.04, $n = 3$–4, $P < 0.05$, Fig. 5B). Moreover, we found different evidence of a mutual interaction between PKGIα and VASP activity. We observed that downregulation of PKGIα expression induced a 20% decrease in the level of VASP protein (1.52 ± 0.06 versus 1.22 ± 0.04, $n = 9$, $P < 0.05$, Fig. 5C) under HG conditions.

This observation raises the question of whether increased phosphorylation of VASP determines a PKGIα-mediated decrease in pMLC in podocytes. To investigate this in more detail, we examined the level of pMLC phosphorylation under the same experimental conditions as in the pVASP Ser239 experiments above. Podocytes with reduced PKGIα gene expression and podocytes treated with Rp-8-Br-cGMPS had the same level of pMLC as control cells cultured under SG conditions (Fig. 5D). Under HG conditions, the pMCL level was diminished by 21% (0.81 ± 0.03 versus 0.64 ± 0.03, $n = 10$–11, $P < 0.05$). In addition, inhibition of PKGIα activity by PKGIα siRNA or Rp-8-Br-cGMPS significantly increased the phosphorylation of MLC (Fig. 5D).

These results indicate a possible mechanism by which HG-dependent activation of the PKGIα/VASP signalling pathway leads to diminished pMLC and contributes to limited podocyte contractility.

Effect of insulin and HG on PKGIα/VASP signalling in podocytes with downregulated VASP expression

We previously showed that insulin and HG contribute to a reduction in the pMLC pool in a PKGIα-dependent manner (*18*). Based on our findings that PKGIα exhibits a regulatory effect on VASP expression, we examined whether VASP is involved in insulin- and HG-dependent regulation of PKGIα expression and the contractile apparatus in podocytes.

Therefore, we used siRNA to downregulate VASP expression in podocytes. Based on obtained results

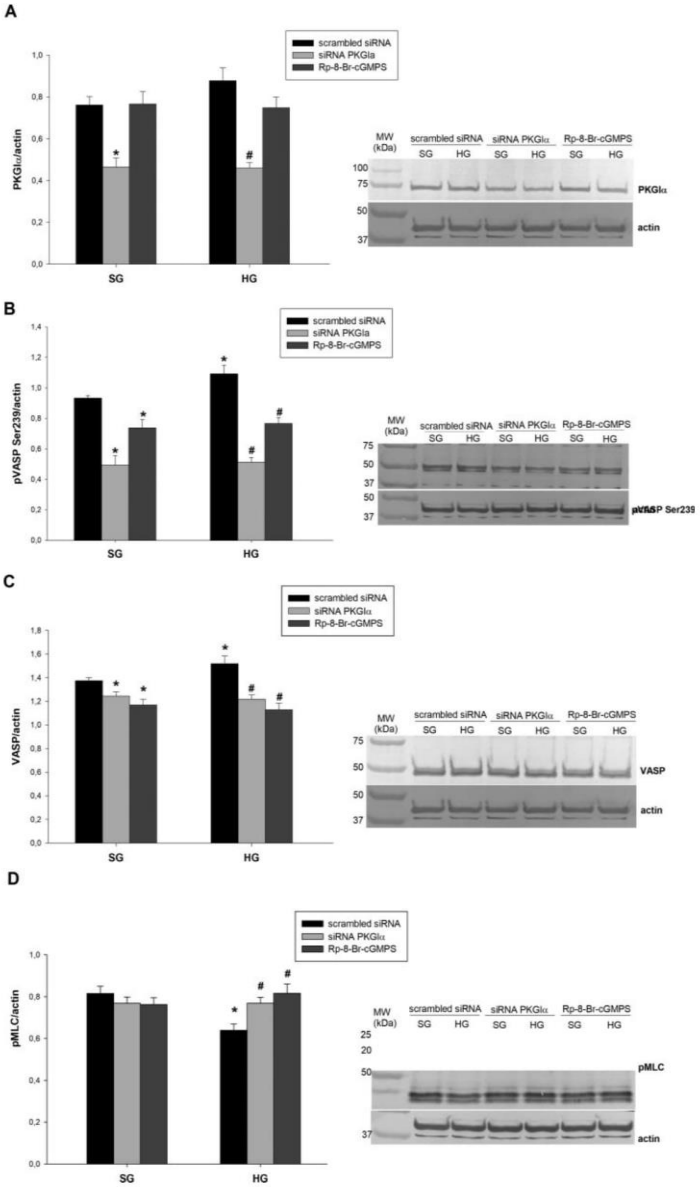

Fig. 5. Effect of PKGIα gene silencing on VASP and pMLC expression in rat podocytes. Podocytes were transfected with PKGIα siRNA and cultured in SG (11 mM) or HG (30 mM) medium in the presence or absence of Rp-8-Br-cGMPS (50 μM) for 5 days. The band intensities for PKGIα (**A**), pVASP Ser239 (**B**), VASP (**C**) and pMLC (**D**) are reported as the ratio to the actin band intensity. Values are the mean ± SEM, $n = 3$–11, $^{*}P < 0.05$ compared with SG scrambled siRNA, $^{\#}P < 0.05$ compared with appropriate scrambled siRNA.

(Fig. 6A), the VASP siRNA Duplex A was used. We observed an ~35% decrease in VASP protein (1.11 ± 0.05 to 0.72 ± 0.03; $n = 11$–13, $P < 0.05$, Fig. 6A) in podocytes. Taking into consideration the fact, that podocytes are difficult to transfect as they constitute primary cell line, the ~35% knockdown of

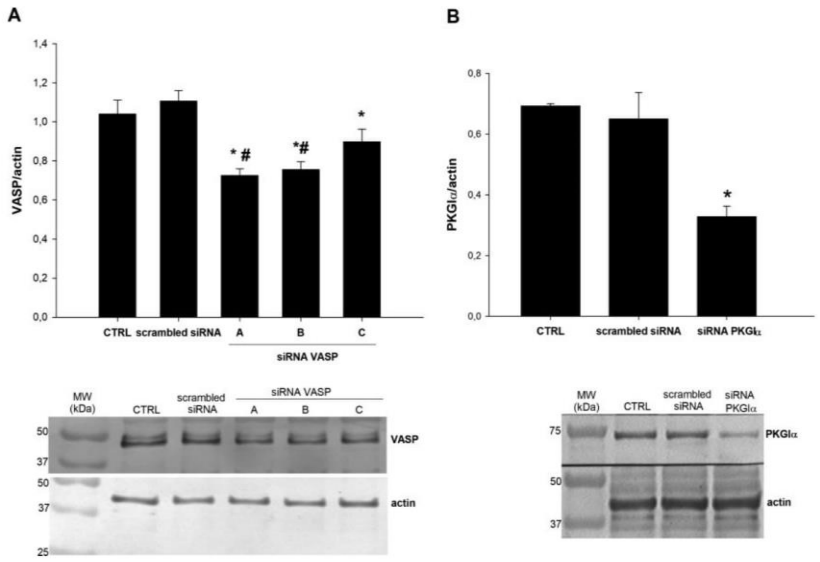

Fig. 6. Effects of siRNA targeting VASP and PKGIα on the expression of VASP and PKGIα proteins in cultured rat podocytes. Densitometric analysis of (A) and (B) was performed to establish the amount of VASP and PKGIα proteins, respectively. Actin was used as a loading control. Controls (CTRL) were run with scrambled siRNA. Values are the mean ± SEM, $n = 11$–13 (VASP) and $n = 3$ (PKGIα), $^{*}P < 0.05$ compared with scrambled siRNA, $^{\#}P < 0.05$ compared with CTRL (non-transfected podocytes).

the VASP expression was considered as satisfactory for further research.

Insulin or HG alone caused increase in the level pVASP Ser239 by 21% (Fig. 7A). The combined effect of insulin and HG on these proteins was not additive. Furthermore, VASP siRNA transfection drastically decreased the level of pVASP Ser239, by 69% in the presence of SG and 77% in the presence of insulin and/or HG (Fig. 7A).

Next, we measured the effect of insulin and/or HG on PKGIα expression after downregulation of VASP using siRNA. The transfection of podocytes with VASP siRNA reduced the effect of both insulin and HG on VASP expression (Fig. 7B). Incubation with insulin and/or HG induced an increase in PKGIα expression in podocytes (Fig. 7C). Interestingly, transfection of podocytes with VASP siRNA substantially reduced the effect of insulin or HG on PKGIα expression, by 24% (1.75 ± 0.05 versus 1.34 ± 0.06, $n = 14$, $P < 0.05$) and 32% (1.9 ± 0.08 versus 1.3 ± 0.06, $n = 14$–15, $P < 0.05$), respectively (Fig. 7C). Notably, VASP downregulation by siRNA significantly diminished the basal PKGIα expression in podocytes (Fig. 7C).

Given the observed effects of insulin and/or HG on PKGIα-dependent phosphorylation of VASP at Ser239, we predicted that the expression of proteins involved in regulating the podocyte contraction apparatus should be restored to a basal level in podocytes transfected with VASP siRNA. We assayed that insulin or HG decreased the basal phosphorylation of MLC (1.49 ± 0.04) by ~13% (Fig. 7D). The combined exposure to insulin and HG had no additive effect on pMLC. In accordance with our predictions, VASP siRNA administration increased pMLC expression by ~23% in the presence of insulin, 21% in the presence of HG, and 39% in the presence of both insulin and HG (Fig. 7D). These results suggest that insulin and HG regulated the podocyte contraction apparatus by signalling to pMLC through activation of the PKGIα/VASP signalling pathway.

Insulin and HG affect actin cytoskeleton organization through a VASP-dependent mechanism in cultured rat podocytes

Because insulin and HG signalling and VASP function are associated with actin filament formation, dynamics, and molecular organization, we hypothesized that VASP mediates actin cytoskeleton reorganization in rat podocytes cultured under hyperinsulinaemic or hyperglycaemic conditions. Podocytes seeded on coverslips were transfected with VASP siRNA and cultured in SG or HG medium in the presence or absence of insulin. Morphological changes of cells were captured using a fluorescence microscope. The F-actin of the podocytes exposed to SG conditions was distributed as prominent bundles that traversed the cell along the axis of the podocyte (Fig. 8). Insulin- and HG-treated cells demonstrated increased F-actin immunostaining in cortical regions, grouped together along the cell periphery, but insulin and HG had only a slight effect on intracellular F-actin staining. The effects of insulin and HG on F-actin network organization were abolished by siRNA-mediated knockdown of VASP expression (Fig. 8).

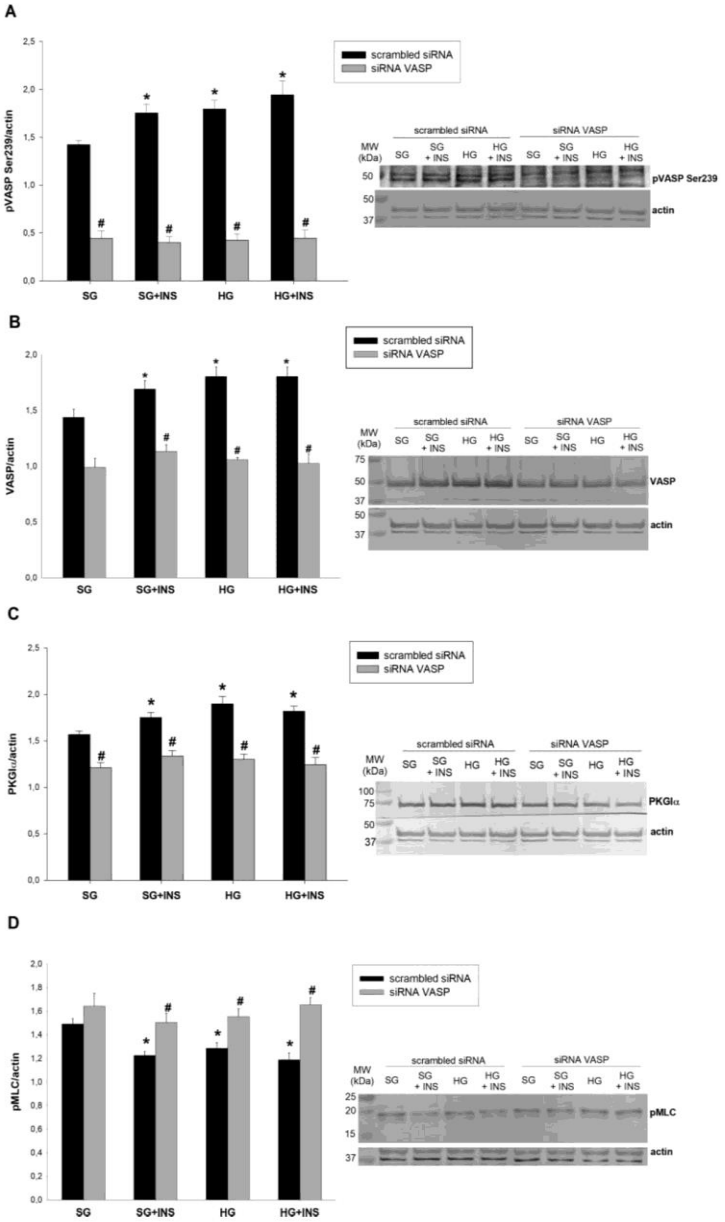

Fig. 7. Effect of VASP gene silencing on PKGIα-dependent pMLC. Cells were transfected with VASP siRNA and cultured under SG or HG conditions in the presence or absence of insulin (INS, 300 nM, 5 days). The band intensities for pVASP Ser239 (**A**), VASP (**B**), PKGIα (**C**) and pMLC (**D**) are reported as the ratio to the actin band intensity. Values are the mean ± SEM, $n = 9$–15. $^{*}P < 0.05$ compared with SG scrambled siRNA, $^{\#}P < .05$ compared with appropriate scrambled siRNA.

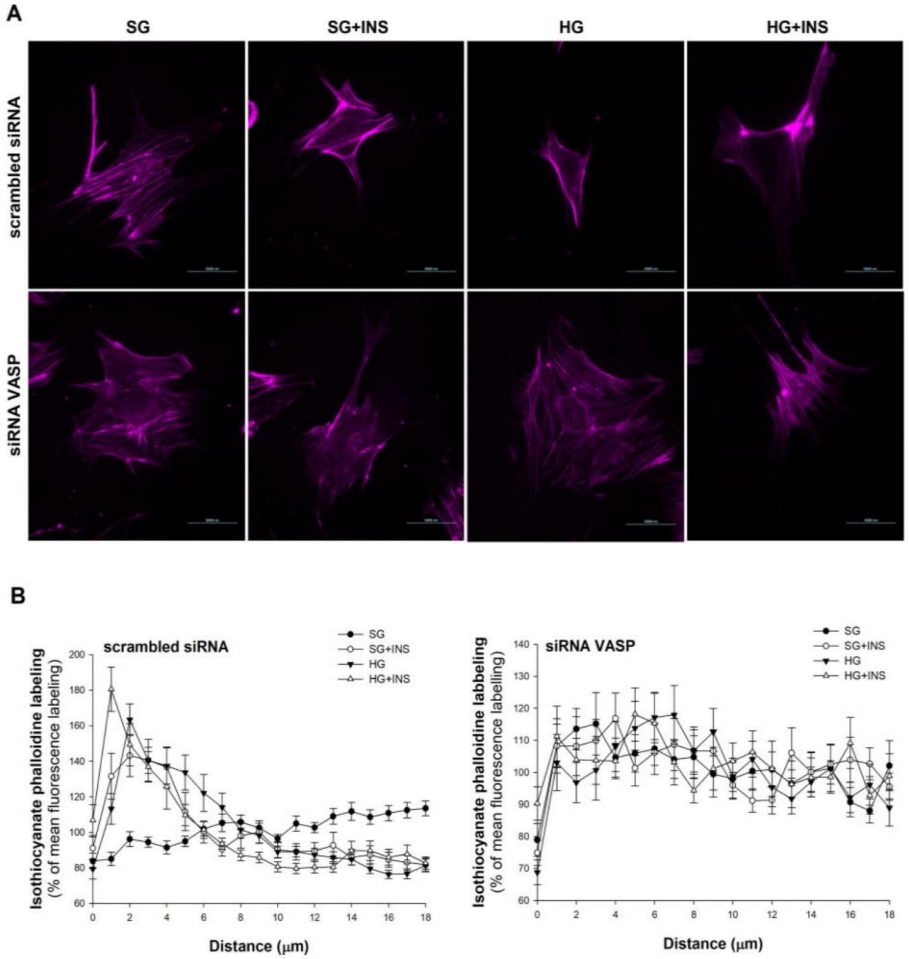

Fig. 8. VASP mediates remodelling of the actin cytoskeleton in podocytes. Podocytes were seeded on coverslips and cultured in SG (11 mM) or HG (30 mM) medium in the presence or absence of insulin (INS, 300 nM) for 5 days. The F-actin network was labeled with isothiocyanate phalloidin (1:200) and visualized by fluorescence microscopy (**A**). The analysis of fluorescence intensity profile (measured from the basal membrane to the nucleus) was performed using NIS-Elements software (Nikon) (**B**). Values are mean ± SEM, $n = 17$–20. Scale bars: 5000 nm.

Insulin and HG increase permeability of cultured rat podocytes to albumin through a VASP-dependent mechanism

In light of recent findings that insulin and HG increase podocyte permeability to albumin in a PKGIα-dependent manner (*18*), we hypothesized that VASP is part of this signalling pathway. Both insulin and HG markedly increased podocyte permeability. The transmembrane flux for albumin markedly increased 43.7% in podocytes incubated with insulin. Moreover, under HG conditions, albumin permeation was significantly elevated, by 40% (Fig. 9). The combined action of insulin and HG had no additional effect on albumin permeability (Fig. 9).

To characterize the role of VASP in the regulation of podocyte permeability to albumin, we knocked down VASP protein expression using siRNA. Cells transfected with VASP siRNA exhibited a significant reduction in podocyte permeability to albumin under both hyperinsulinaemic (32.53 ± 2.72 versus 55.56 ± 3.02 μg/ml, $n = 13$–14, $P < 0.05$) and hyperglycaemic (32.83 ± 2.43 versus 52.18 ± 4.43 μg/ml, $n = 12$–14, $P < 0.05$) conditions (Fig. 9). These results suggest that insulin- and HG-triggered PKGIα/VASP

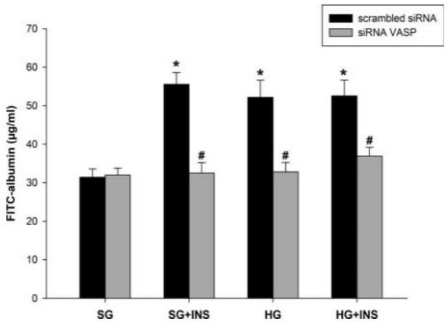

Fig. 9. Effect of VASP on the insulin- and HG-dependent increase in podocyte permeability to albumin. Transepithelial permeability to albumin was assessed by measuring the diffusion of FITC-labelled albumin across the podocyte monolayer. Podocytes were cultured in SG (11 mM) medium or HG (30 mM) medium in the presence or absence of insulin (INS, 300 nM) for 5 days. Values are mean ± SEM, $n = 12$–14. $^{*}P < 0.05$ compared with SG scrambled siRNA, $^{\#}P < 0.05$ compared with appropriate scrambled siRNA.

signalling to the actin cytoskeleton, resulting in its reorganization and increases permeability to albumin across the podocyte filtration barrier.

Discussion

This study revealed a new mechanism for the regulation of podocyte barrier permeability through PKGIα-dependent phosphorylation of VASP at Ser239 under hyperinsulinaemic and hyperglycaemic conditions. Activation of the PKGIα/VASP pathway may disturb the permeability of the glomerular filtration barrier and mediate the development of DN. The proposed mechanism is shown in Fig. 10.

Short incubation with insulin rapidly increased PKGIα-dependent pVASP Ser239 in podocytes. Next, long-term incubation with insulin and HG induced a decrease of VASP Ser239 phosphorylation via PKGIα-dependent manner in cultured rat podocytes. VASP mediated reorganization of the actin cytoskeleton in podocytes cultured under hyperinsulinaemic or hyperglycaemic conditions, and insulin and HG increased podocyte barrier permeability through stimulation of VASP signalling pathways.

Podocytes regulate glomerular filtration barrier permeability via their contractile properties, which are closely related to dynamic actin cytoskeleton reorganization. A high concentration of insulin- and glucose-mediated podocyte injury, which altered FP actin organization, resulting in increased glomerular barrier permeability (*18*, *40*). We showed that both insulin and HG mediate regulation of the contractile apparatus and filtration barrier permeability through activation of PKGIα signalling pathways in cultured rat podocytes (*18*, *37*). This study confirmed the pivotal role of PKGIα in regulation of the contractile apparatus in podocytes under hyperinsulinaemic and hyperglycaemic conditions. Furthermore, for the first time, we demonstrated that VASP interacts with PKGIα and is involved in the regulation of actin organization

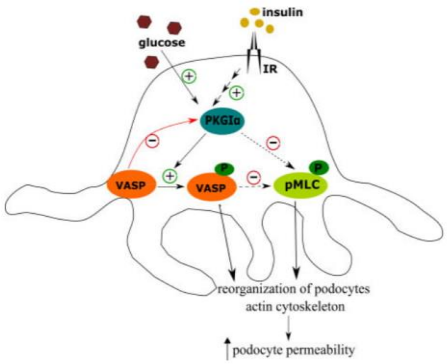

Fig. 10. The proposed mechanism for podocyte permeability through insulin- and HG-dependent activation of the PKGIα/VASP signalling pathway in cultured rat podocytes.

and albumin permeability in cultured rat podocytes in response to insulin and HG.

It is thought that insulin induces a dynamic reorganization of podocyte FPs after meal to deal with transiently elevated filtration load (*12*). Our recent studies showed that insulin induces PKGIα activation and increases permeability of both podocytes and glomeruli (*14*, *37*). Therefore, we examined the contribution of insulin in PKGI-dependent phosphorylation of VASP at Ser239 in podocytes. We demonstrated that short exposure of cells to either insulin or cGMP analogue 8-Br-cGMP quickly increases pVASP Ser239, and this effect was abolished by the PKG inhibitor. Moreover, immunofluorescent staining confirmed that the short-term incubation of podocytes with insulin significantly increases the interaction between PKGIα and VASP. These results confirmed that phosphorylation of VASP at Ser239 is PKGIα specific.

Based on our findings that disulphide activation of PKGIα is a relevant factor that increases podocyte permeability to albumin during hyperinsulinemia (*14*) and hyperglycaemia (*19*), we researched the effect of insulin and HG on VASP expression, its phosphorylated state and localization in podocytes. This study demonstrated that insulin and HG augment VASP expression and change its localization in podocytes. Perhaps diabetic conditions alter the VASP phosphorylation state, which in turn may change VASP localization and affect actin filament dynamics. The level of pVASP Ser239 protein was also significantly elevated by insulin and HG, which is consistent with increased expression of PKGIα under the same experimental conditions. Inhibition of PKGIα activity by specific gene silencing or Rp-8-Br-cGMPS decreased the amount of total VASP and its phosphorylated form in podocytes, confirming the interaction between VASP and PKGIα. We also found that podocyte transfection with VASP siRNA decreased pVASP Ser239 under hyperinsulinaemic and hyperglycaemic conditions. Surprisingly, VASP gene silencing significantly diminished the basal level of PKGIα protein in insulin- and HG-stimulated podocytes, suggesting

that a negative feedback regulatory mechanism between VASP and PKGIα exist. In another study, HG treatment resulted in an opposite effect on pVASP Ser239 in VSMCs. However, the experiment incorporated different cell types, duration of incubation with HG, and assumed impairment of cGMP-mediated pVASP Ser239 through oxidative stress (*41*). In podocytes, oxidative stress activates PKGIα independently of cGMP signalling (*17*).

PKGIα is implicated in the regulation of the podocyte contractile apparatus via MLC phosphatase-dependent dephosphorylation of MLC at Ser19 (*16*, *17*). In this study, we demonstrated that insulin and HG regulate the podocyte contraction apparatus through activation of the PKGIα/VASP signalling pathway. siRNA-mediated knockdown of VASP abolished the effect of insulin and HG on pMLC. Therefore, active PKGIα phosphorylated VASP at Ser239, resulting in signal transduction to the contractile apparatus, and may promoted dephosphorylation of MLC under diabetic conditions. In VSMCs, relaxation was also determined by PKG/VASP signalling, including increased pVASP Ser239 (*42*, *43*). Studies performed on mouse VASP-deficient cardiac fibroblasts showed that basal pMLC is significantly increased (*22*).

Podocyte function as main regulator of glomerular filtration barrier permeability relies on dynamic reorganization of the actin cytoskeleton. More than 100 proteins are involved in controlling actin dynamics (*44*), including VASP, which acts as the actin filament elongation factor (*28*). In VSMCs, knockdown of VASP diminished cell contractility, which was associated with limited elongation of actin filaments (*29*). In this study, we demonstrated that insulin and HG induce filamentous actin (F-actin) reorganization by affecting VASP function. siRNA-mediated knockdown of VASP prevented the accumulation of F-actin close to the plasma membrane in podocytes cultured in medium supplemented with a high concentration of insulin or glucose. Recent studies have shown that Ena/VASP proteins reduce F-actin branching, and actin filaments are longer (*45*). These results suggest that VASP signalling to the actin cytoskeleton may be part of a significant dysfunctional pathway in insulin-resistant states. In addition, podocytes incubated with nephrotic plasma have increased VASP phosphorylation and enhanced cell migration, which is associated with dynamic actin remodelling (*33*). In another study, authors suggested that NO-induced pVASP Ser239 leads to drastically decreased F-actin in cell processes of PTECs, retraction of lamellipodia, and cell rounding (*46*). Thus, the opposite effect of VASP phosphorylation on cell migration may be cell type-dependent and differ due to activators and activated pathways.

Improper podocyte cytoskeletal reorganization causes not only insulin resistance, but also increased albumin permeability. Our findings indicate that VASP is involved in the insulin- and HG-dependent increase in podocyte permeability to albumin. Downregulation of VASP expression greatly reduced the albumin permeability induced by high concentrations of both insulin and glucose. Insulin and HG had no synergistic effect on podocyte permeability to albumin. It may indicate that the main role in insulin- and HG-dependent regulation of podocyte permeability plays PKGIα/VASP pathway. Combined usage of insulin and HG had no additional effect also on PKGIα protein expression and on the phosphorylation of MLC. Moreover, these experimental conditions did not affect further increase of pVASP Ser239. Piwkowska *et al.* (*18*) have previously showed that simultaneous treatment of podocytes with insulin and HG does not have additive effects on PKGIα and MLC activation or expression, compared with both insulin and HG alone. However, they hypothesized that insulin- and HG-dependent activation of PKGIα may be responsible, at least partially, for an increased podocyte permeability to albumin. Studies conducted on brain endothelial cells have shown that increased pVASP Ser239 is associated with an increased blood-brain barrier permeability under hypoxic conditions (*47*). Davis *et al.* (*47*) has also suggested that VASP phosphorylation followed hypoxia is mediated by activation of vascular endothelial growth factor (VEGF) and its receptor VEGFR2. In podocytes, insulin stimulates the production of VEGF-A (*48*), which is an important autocrine factor that preserves the integrity of the glomerular filtration barrier through a modification of podocyte structure and function (*49*, *50*). Unfortunately, the role of VASP phosphorylation in changes of podocyte permeability induced by VEGF-A is largely unknown. In pulmonary artery endothelial cells, VASP phosphorylation has a protective effect on barrier permeability (*51*). However, this effect is mediated by cGMP, which is thought to indicate a protective influence on endothelial barrier function. In hyperglycaemic conditions, the concentration of cGMP decreased (*52–54*), and this may be a cause of podocyte injury. Moreover, Hohenstein *et al.* (*55*) showed that, in podocytes, VASP expression is significantly increased during crescentic nephritis and correlates with a loss of podocytes. The data suggest that enhanced VASP expression may play an important role in podocyte pathophysiology and impair the function of the glomerular filtration barrier. Unfortunately, the underlying mechanism in the regulation of podocyte permeability is less understood and could involve changes in actin organization mediated by VASP phosphorylation. Therefore, the precise regulatory mechanisms between VASP and PKGIα and the role of VASP phosphorylation by PKG in the control of podocyte function under diabetic conditions will require further study.

In conclusion, the results suggest that the insulin- and HG-induced increase in podocyte barrier permeability may be due to activation of PKGIα/VASP signalling to the actin cytoskeleton. This mechanism may be responsible for increased glomerular filtration barrier permeability, resulting in albuminuria in patients suffering from elevated insulin or glucose.

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Conflict of Interest
None declared.

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REVIEW

The role of vasodilator-stimulated phosphoprotein in podocyte functioning

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Abstract

Vasodilator-stimulated phosphoprotein (VASP) is a 39-kDa protein belonging to the Ena/VASP protein family, which is involved in adhesion, migration, cell–cell interaction, and regulation of pathways connected with actin cytoskeleton remodeling. VASP is phosphorylated at Tyr39, Ser157, Ser239, Thr278, and Ser322 mainly by tyrosine kinase Abl, cAMP-dependent protein kinase, protein kinase G, AMP-activated protein kinase, and protein kinase D1, respectively. VASP phosphorylation, as a regulator of actin dynamics, may lead to impaired reorganization of the podocyte actin cytoskeleton not only by indirect interaction of VASP with actin but also by regulation of other signaling pathways. A few studies have shown that VASP participates in the development of renal diseases and mediates podocyte movement through its interaction with proteins of the slit diaphragm. VASP phosphorylation may cause reduced actin filament assembly in podocytes and mediate disturbances in regulation of filtration barrier permeability as a consequence of podocyte foot process effacement. In this paper, we describe the role of VASP in podocyte function, mainly in the context of actin dynamics and glomerular filtration barrier permeability. In addition, we discuss the involvement of VASP and its phosphorylated forms in the development of kidney diseases.

Keywords: actin cytoskeleton; filtration barrier permeability; podocyte; slit diaphragm; vasodilator-stimulated phosphoprotein

Introduction

Kidneys are among the most important excretory organs and responsible for eliminating waste products from the blood (Kokot, 1975). This function is possible thanks to the co-operation of many specialized cells that form the glomerulus, the filtering unit of the kidney. The glomerulus is a network of capillaries supported by intraglomerular mesangial cells, all enclosed in the Bowman's capsule (Pollak et al., 2014). The glomerular filtration barrier consists of the fenestrated endothelial cells, glomerular basement membrane (GBM), and podocytes (Arif and Nihalani, 2013). Podocytes are highly specialized cells consisting of a cell body, major foot processes, and interdigitated foot processes that completely cover glomerular capillaries and form filtration slits (Reiser and Altintas, 2016). The most important structure of the filtration barrier is the slit diaphragm (SD) that extends across the filtration slit and connects neighboring podocyte foot processes (Reiser and Altintas, 2016). The SD is a modified adherent junction that forms a signaling platform to regulate podocyte function and morphology (Huber and Benzing, 2005). The SD is composed of many proteins, including nephrin, podocin, TRPC6, and actin. Mutations in these proteins have been tied to diseases associated with foot process effacement and proteinuria (Fukasawa et al., 2009; Moller et al., 2009).

Foot process effacement is the retraction of foot processes and results from podocyte injury that alters actin cytoskeleton structure (Kriz and Lemley, 1999). The effacement involves the active rearrangement of actin filaments, regulated by proteins associated with the podocyte actin cytoskeleton. The precise control of actin

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Abbreviations: Abl, tyrosine kinase Abl; ActA, actin assembly-inducing protein; AMPK, AMP-activated protein kinase; CP, capping protein; EVH1, Ena/VASP homology domain 1; EVH2, Ena/VASP homology domain 2; Evl, Ena/VASP-like protein; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; PAR1, protease-activated receptor-1; PKA, cAMP-dependent protein kinase; PKD1, protein kinase D1; PKG, protein kinase G; PP, protein phosphatases; PRR, proline-rich region; SD, slit diaphragm; SOC, store-operated Ca^{2+} channel; TRPC6, transient receptor potential canonical channel 6; VASP, vasodilator-stimulated phosphoprotein; wt, wild type

organization is essential for maintaining the proper function of the cytoskeleton and its specialized structures in vivo. Assembling/disassembling of actin filaments and their spatial organization are coordinated by numerous actin-binding proteins (Quinlan, 2004; Disanza et al., 2005). Among these are vasodilator-stimulated phosphoprotein (VASP), a 39-kDa protein of the Ena/VASP family of actin-regulatory proteins, which share homology with mammalian Mena (the homologue of *Drosophila* Ena) and Evl (Ena/VASP-like protein) (Laurent et al., 1999).

VASP was first characterized in endothelial cells and platelets (Halbrügge and Walter, 1989). In addition, VASP is highly expressed in spleen, stomach, intestine, lung, and kidney, but its exact role in these tissues is unknown (Gambaryan et al., 2001).

VASP contains three domains: N-terminal Ena/VASP homology domain 1 (EVH1), a central proline-rich region (PRR), and C-terminal Ena/VASP homology domain 2 (EVH2) (Chereau and Dominguez, 2006; Blume et al., 2007). EVH1 is responsible for binding VASP to the proline-rich motifs of proteins associated with the cytoskeleton, such as vinculin, zyxin, lamellipodin, and actin assembly-inducing protein (ActA), which is the protein of the intracellular bacterial pathogen *Listeria monocytogenes* (Callebaut et al., 1998; Hansen and Mullins, 2015; Acevedo et al., 2017). The PRR contains binding sites for profilin and proteins containing WW (named for a conserved Trp-Trp motif) and Src homology 3 (SH3) domains (Holt et al., 1998; Krause et al., 2003). The EVH2 domain contains a G-actin–binding site, an F-actin–binding site, and a coiled-coil motif required for oligomerization of Ena/VASP proteins (Bachmann et al., 1999; Harbeck et al., 2000).

VASP plays a key role as a molecular adaptor-related to dynamic membrane activity and is involved in cell adhesion and migration (Wu and Gunst, 2015). Moreover, it acts as a regulator of proteins associated with remodeling of the actin cytoskeleton (Hütte lmaier et al., 1999; Harbeck et al., 2000; Walders-Harbeck et al., 2002; Galler et al., 2006; Benz et al., 2009). In this paper, we describe the role of VASP in podocyte functioning, including how changes in VASP phosphorylation can affect the development of glomerular diseases through the remodeling of the actin cytoskeleton in podocytes.

Actin dynamics and its role in podocyte functioning

The actin cytoskeleton is a complex organization of actin filaments (F-actin) with their accessory and regulatory proteins, which is the main source of force-generating machinery in the cell (Blanchoin et al., 2014; Svitkina, 2018). The cytoskeleton plays a key role in the production of contractile forces through the interaction of actin filaments with myosin II, and in cell division, facilitation of harmonized cell movement driven by filopodia and lamellipodia, and coordination of endocytosis and phagocytosis (Arber et al., 1998; Mooren et al., 2012). Moreover, the actin cytoskeleton is responsible for maintaining cell shape and the mechanical features of the cell surface, driving the intracellular movement of membrane organelles and providing cell–cell adhesions and interaction with the extracellular matrix (Galler et al., 2006; Trichet et al., 2008; Svitkina, 2018).

The cytoskeleton also determines podocyte shape and function. The cell body consists mainly of microtubules and vimentin intermediate filaments, whereas foot processes contain thick bundles of F-actin surrounded by branched actin filaments that run cortically and contiguously connect to neighboring processes (Noris and Remuzzi, 2012; Neal, 2015). This composition is tied to function. Podocyte foot processes link these cells and attach them through focal adhesions to the GBM, whereas beneath the podocyte cell body or its processes there is a space called the subpodocyte space (Neal et al., 2005; Neal et al., 2007; Kriz et al., 2013). Reorganization of the actin cytoskeleton is considered to be the contractile force that mediates active modulation of filtration barrier permeability through alterations in foot process morphology. Podocyte injury or stress leads to the formation of a dense network of dislocated filaments and is associated with foot process effacement characterized by the loss of the interdigitating pattern of adjacent foot processes and of the SD (Noris and Remuzzi, 2012; Kriz et al., 2013). Foot process effacement is considered to be the podocyte's compensatory and adaptive mechanism to protect cells against detachment. The loss of mechanisms responsible for maintaining attachment leads to the detachment of a viable podocyte from the GBM and it is excreted in the urine (Vogelmann et al., 2003).

The SD is connected to the podocyte actin cytoskeleton through multiple adaptor proteins, including α-actinin, CD2AP, synaptopodin, and zonula occludens-1 (Faul et al., 2007). Proteins that are essential for the proper functioning of SD include nephrin and podocin. Nephrin is a transmembrane protein with a short intracellular portion and an extracellular segment with IgG- and fibronectin-type III–like motifs. This protein interacts with the podocyte actin cytoskeleton and serves as a scaffold for the SD (Patrakka and Tryggvason, 2007). Nephrin phosphorylation leads to the recruitment of Nck protein to the intracellular domain and promotion of localized actin polymerization (Lehtonen, 2008). At the SD, nephrin interacts with podocin, which is an integral membrane protein and forms highly organized oligomeric structures associated with lipid rafts (Shono et al., 2007).

There is a strong relationship between nephrin, podocin and the podocyte actin cytoskeleton, where both nephrin

and podocin are involved in foot process formation by affecting actin filaments (Saleem et al., 2002). Loss of nephrin or podocin leads to proteinuria, a manifestation of foot process effacement (Kestilä et al., 1998; George and Holzman, 2012). Evidence shows that nephrin and podocin are associated with the actin cytoskeleton and affect its organization, but the exact mechanism is unknown. However, an earlier report (Harris et al., 2013) has indicated that podocin interacts with VASP, which may influence actin dynamics and actin cytoskeleton reorganization.

The role of VASP in regulating actin dynamics

Actin filament consists of globular actin that undergoes continuous assembly and disassembly during the mechanism of treadmilling (Lee and Dominguez, 2010). In this process, the filamentous actin is elongated by reversible addition of actin monomers (G-actin) to the fast polymerizing end (barbed end) faster than an addition to the slow polymerizing end (pointed end). The actin monomers with adenosine triphosphate bind to the barbed end of the existing actin filament, while monomers with adenosine diphosphate are detached from the pointed end (Cooper and Sunderland, 2000; Schüler et al., 2006; Interliggi et al., 2007).

One of the functions attributed to VASP is the anti-capping activity that allows for elongation of the actin filament, even if capping proteins (CPs) are present. This mechanism is based on associating Ena/VASP proteins with elongating F-actin at/near their barbed end in a way that inhibits CP action (Bear and Gertler, 2009). An earlier study (Breitsprecher et al., 2008) confirmed the anti-capping function of VASP, which can support continuous F-actin elongation even in the presence of CP. Moreover, (Applewhite et al., 2007) suggested a hypothesis that filopodia formation is initiated by Ena/VASP proteins. In the beginning, the Ena/VASP proteins function to cross-link or aggregate barbed ends of actin through tetramerization and at the same time Ena/VASP interacts with F-actin (Applewhite et al., 2007). This is consistent with the fact that Ena/VASP is able to bundle F-actin (Bachmann et al., 1999; Hüttelmaier et al., 1999; Laurent et al., 1999). In the next step, Ena/VASP is temporarily recruited to the membrane-bound ligands located on the leading edge to stabilize the full-length F-actin at filopodial tips (Applewhite et al., 2007). To allow for continuous polymerization by the insertion of G-actin, the interaction between GAB motif of Ena/VASP and F-actin barbed ends is transient in filopodia (Applewhite et al., 2007).

Ena/VASP proteins also can nucleate F-actin in vitro. The Ena/VASP-dependent nucleation of F-actin was confirmed through using pyrene-actin polymerization assay (Hüttelmaier et al., 1999; Skoble et al., 2001). However, in vivo, it is unlikely that VASP is a physiological nucleator of actin because this process is extremely dependent on salt content and usually takes place at subphysiological salt concentrations (Bear and Gertler, 2009).

Furthermore, numerous studies have shown that Ena/VASP proteins restrict F-actin branching by Arp2/3 complex (Bear et al., 2002; Samarin et al., 2003; Sechi and Wehland, 2004); however, the Ena/VASP direct effect on branching is unknown (Skoble et al., 2001; Bear and Gertler, 2009). It is hypothesized that Ena/VASP proteins compete with the Arp2/3 complex for actin monomers (essential cofactors for branching) (Bear and Gertler, 2009). Another likely hypothesis for anti-branching ability of Ena/VASP is that increased CP activity augments branching as a result of locally increasing levels of actin monomers (Akin and Mullins, 2008). It is predicted that Ena/VASP with its anti-capping function may decrease branching by the diminished capping of barbed ends, which could lead to increased consumption of G-actin by its addition to barbed ends (Bear and Gertler, 2009). Unfortunately, any of these mechanisms have not been investigated in podocytes, so to date, this issue remains unresolved.

Regulation of VASP function

VASP phosphorylation depends on intracellular localization, the availability of phosphorylation sites in VASP for other proteins, accessibility of particular protein kinases and/or protein phosphatases (PP), and the individual activators and inhibitors of these phosphatases and kinases (Calzi et al., 2008). In addition, phosphorylation is a convenient mechanism of control that is fast and effective, especially with a protein that is constitutively present within cells.

VASP has five identified phosphorylation sites: Tyr39, Ser157, Ser239, Thr278, and Ser322 (Figure 1) (Döppler and Storz, 2013). The function of VASP is regulated mainly by serine/threonine kinases such as cAMP-dependent protein kinase (PKA), protein kinase G (PKG), AMP-activated protein kinase (AMPK), and protein kinase D1 (PKD1). A few years ago tyrosine kinase Abl (Abl) joined this group (Thomson et al., 2011; Maruoka et al., 2012; Döppler and Storz, 2013).

VASP is phosphorylated by PKA at Ser157 with faster kinetics than Ser239 and Thr278. Phosphorylation of VASP at Ser157 leads to changes in VASP localization within the cell without affecting actin filament formation or the G-actin/F-actin ratio and inhibits VASP interaction with the SH3 domains of Abl kinase and nSrc (Harbeck et al., 2000; Pula and Krause, 2008; Benz et al., 2009). Phosphorylation of VASP at this site (Ser157) is probably necessary for targeting VASP to its potential functional sites within the

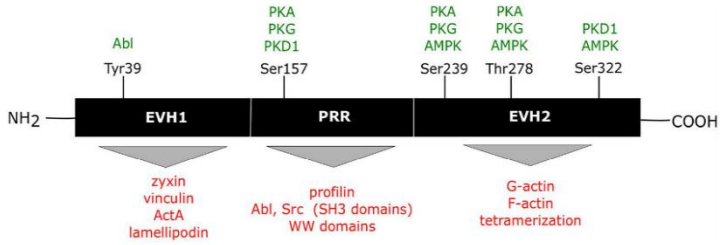

Figure 1 Organization of human vasodilator-stimulated phosphoprotein (VASP), localization of phosphorylation sites, and overview of proteins interacting with VASP (Döppler and Storz, 2013; modified).

cell (Benz et al., 2009). VASP is also phosphorylated by PKD1 at Ser157 (Döppler et al., 2013). Protein kinase C and Rho-associated protein kinase 1 are upstream of PKD1 in RhoA-signaling pathways, and it is probable that PKD1 contributes to protein kinase C–dependent VASP phosphorylation (Döppler and Storz, 2013).

As noted, VASP is also a substrate for PKG, which in the first instance phosphorylates Ser239 and then Ser157 (Smolenski et al., 1998; Zhang et al., 2010). Studies by Routray et al. (2011) revealed that phosphorylation of Ser157, Ser239, and Thr278 through PKG disturbs Rac1-dependent focal adhesion assembly in hepatic stellate cells.

The next VASP phosphorylation site is Thr278. This amino acid is phosphorylated by both PKA and PKG, but only following phosphorylation of VASP at Ser157 and Ser239. A recent study has shown that AMPK prefers Thr278, and this reaction causes reduced F-actin assembly and consequently impaired stress fiber formation and altered cell morphology (Blume et al., 2007). Another newly characterized phosphorylation site preferred by AMPK and PKD1 is Ser322. Phosphorylation at this site increases F-actin accumulation and promotes filopodia formation (Thomson et al., 2011; Döppler et al., 2013).

Abl is another kinase that phosphorylates VASP at Tyr39 and limits VASP localization in focal adhesions (Maruoka et al., 2012). Moreover, Abl is one of the activators of PKD1 during oxidative stress. It is possible that Abl regulates VASP function by its phosphorylation at Tyr39 in a PKD1-dependent manner, an issue worth investigating (Döppler and Storz, 2013).

Little is known about the mechanisms of VASP dephosphorylation. This process has been studied only in platelets, where inactivation of VASP is implemented by PPs: PP2A, PP2B, and PP2C in vitro (Abel et al., 1995). PP2A completely dephosphorylates VASP, whereas PP2B and PP2C dephosphorylate VASP at Ser239 and Thr278 sooner than at Ser157 (Abel et al., 1995).

Phosphorylation and dephosphorylation are important regulatory mechanisms of VASP function that affect the actin cytoskeleton. Understanding these patterns allows for defining the pathological mechanisms responsible for disturbances in actin organization and disease development.

The role of VASP in podocytes

Experimental evidence shows that VASP is expressed in the kidney, including mesangial cells, myofibroblasts in the cortex, interstitial cells, and podocytes (Gambaryan et al., 2001; Hohenstein et al., 2005). Unfortunately, the exact role of VASP and its phosphorylation in the whole kidney and particular cells of a healthy kidney, especially in podocytes, is not known. Only a few experiments have indicated the role of VASP in podocyte function.

The role of VASP in vivo has been studied in induced passive crescentic nephrotoxic nephritis. Kidneys of healthy VASP-deficient and wild-type (wt) mice were compared with kidneys of VASP-deficient and wt mice with the disease, respectively (Hohenstein et al., 2005). In healthy mice, VASP was found in blood vessel walls and in interstitial and glomerular mesangial cells. During nephritis, VASP expression markedly increased in podocytes. Progression of nephrotoxic nephritis in wt mice after disease induction was characterized by glomerulosclerosis, glomerular injury, and loss of podocytes. Progression of chronic renal disease observed in wt mice could also be observed but to a lesser degree in VASP-deficient mice, in which kidney's structures like glomeruli and podocytes were significantly protected against the nephrotoxic nephritis for a long time (Hohenstein et al., 2005). The active rearrangement of the actin cytoskeleton is involved in the process of podocyte detachment, whereas VASP is a regulator of actin dynamics (Faul, 2014). Unfortunately, there are no data about sites of VASP phosphorylation in podocytes with nephrotoxic nephritis and its influence on disease development. It is possible that the loss of podocytes during nephrotoxic nephritis is the result of VASP phosphorylation, which may impair the formation of F-actin and may contribute to the effacement of foot

processes. The results of other studies have shown that VASP phosphorylation at Ser239 (pVASP Ser239) and Thr278 (pVASP Thr278) leads to alteration in actin filament accumulation (Harbeck et al., 2000; Benz et al., 2009). Studies by Blume et al. (2007) also indicated that pVASP Thr278 negatively regulates F-actin assembly and reduces the formation of stress fibers. Diminished formation of stress fibers that support podocyte foot processes leads to foot process effacement and may be the reason for proteinuria (Saleem et al., 2002).

The protease-activated receptor-1 (PAR1), like other PARs (PAR2, PAR3, PAR4), is a transmembrane receptor that belongs to the family of G protein-coupled receptors and is activated in response to proteolytic cleavage induced by agonist proteinases (Hirano and Kawabata, 2008). Thrombin is a serine protease that cleaves the N-terminal extracellular region of PAR1 and this cleaved part interacts with the PAR1 to induce transmembrane signaling (Hirano and Kawabata, 2008). Human podocytes express PAR2, PAR3, and PAR4, whereas rat podocytes express all PARs (Sharma et al., 2017). Sharma et al. (2017) demonstrated that the generation of thrombin in plasma increases during nephrotic syndrome and inhibition of this enzyme leads to a decrease in proteinuria in experimental nephrotic syndrome. Moreover, thrombin mediates reorganization of the actin cytoskeleton and induces injury to rat podocytes in a PAR1- and PAR4-dependent manner (Sharma et al., 2017). Recent studies have demonstrated that PAR1 expression of messenger RNA in a mouse model of nephropathy and diabetic nephropathy is significantly elevated (Waasdorp et al., 2016; Guan et al., 2017). Furthermore, inhibition of PAR1 reduces albuminuria and podocyte injury in vivo in the mouse model of streptozotocin-induced diabetic nephropathy (Guan et al., 2017).

PARs may be involved in signaling to the actin cytoskeleton. A recent study has demonstrated that PAR1 signaling to VASP depends on the correct localization of podocin (Harris et al., 2013). They showed that in podocytes incubated with FSGS (focal segmental glomerulosclerosis) plasma containing proteases, VASP is phosphorylated at Ser157 and Ser239 in response to PAR1. In contrast to these data, in podocin-mutant podocytes, PAR1 does not phosphorylate VASP. Moreover, podocytes treated with nephrotic plasma are characterized by increased VASP phosphorylation and enhanced cell migration (Harris et al., 2013). In summary, activation of PAR1 promotes VASP phosphorylation through podocin and increases podocyte motility.

Similar results were reported in endothelial cells, in which VASP phosphorylation at Ser157 also increases their motility (Zhang et al., 2010). These results suggest that VASP phosphorylation is associated with the actin cytoskeleton and its reorganization. Furthermore, VASP-dependent actin reorganization can be regulated by podocin, which is an integral component of the SD. Disturbances in SD functioning may impair signal transduction to the actin cytoskeleton, and podocytes may not be able to properly regulate filtration barrier permeability in response to the signal.

Like podocin, FAT1 is a part of the SD and belongs to the cadherin superfamily (Yaoita et al., 2005). Studies by Moeller et al. (2004) have indicated that FAT1 binds to Ena/VASP proteins and promotes actin polymerization at the leading edge of kidney epithelial cells. The same mechanism may be present in podocytes and regulate podocyte actin dynamics.

Another important component of the SD is transient receptor potential canonical channel 6 (TRPC6), which is an important calcium channel in podocytes (Reiser et al., 2005; Winn et al., 2005). Data show that TRPC6 is associated with the podocyte actin cytoskeleton, which is reorganized after TRPC6 overexpression or insulin treatment (He et al., 2013; Rogacka et al., 2017). We showed that in insulin-treated podocytes, calcium influx is attenuated by inhibition of TRPC6 activity through activation of protein kinase G Iα (PKGIα) (Rogacka et al., 2017). Moreover, insulin plays an important role in glucose uptake, it regulates actin organization in podocytes and is involved in preserving the integrity of the glomerular filtration barrier (Welsh et al., 2010; Coward and Fornoni, 2015). Knocking down of insulin receptor in mice podocytes leads to the loss of their foot processes. Furthermore, these mice develop albuminuria concomitantly with changes typical for diabetic nephropathy, which shows the contribution of insulin signaling to the regulation of the glomerular permeability (Welsh et al., 2010).

In our papers, we demonstrated that insulin increases filtration barrier permeability through TRPC6-dependent activation of PKGIα signaling pathways in podocytes (Piwkowska et al., 2013; Piwkowska et al., 2015; Rogacka et al., 2017). Maybe VASP is involved in this process by phosphorylation of this protein by PKGIα. We noted that PKGIα is localized perinuclear and VASP seems to be less evenly distributed in control podocytes than in cells after insulin treatment. Moreover, the intensity of VASP signal is markedly higher after incubation of podocytes with insulin than in the control cells. Additionally, for the first time we showed that VASP significantly colocalizes with PKGIα after insulin treatment (from 29.72% ± 0.02% to 82.05% ± 0.15%, $n = 3$, $P < 0.05$, paired t student, Figure 2). These results may suggest that insulin regulates podocyte permeability through rearrangement of the actin cytoskeleton as a consequence of VASP phosphorylation in a PKGIα-dependent manner.

Studies conducted on glomerular mesangial cells indicated that TRPC4, a protein in the same family as TRPC6, is negatively regulated by activation of PKGIα. Of note, VASP is involved in this response (Wang et al., 2007). Phosphorylation of VASP at Ser239 by PKGIα leads to interaction between pVASP Ser239 and TRPC4. VASP does not interact with TRPC4 if it is not phosphorylated (Wang et al., 2007). These authors inferred that the association of pVASP Ser239 with TRPC4 is responsible for TRPC4 inhibition, probably by dissociation of pVASP Ser239-TRPC4 from the SOC (store-operated Ca^{2+} channel) complex (O'Neil, 2007). It is highly probable that the same mechanism connected to VASP-dependent regulation of the SOC complex may occur in podocytes and affect the regulation of filtration barrier permeability.

Additionally, VASP seems to be responsible for modulating signaling at the actin cytoskeleton (Walders-Harbeck et al., 2002; Galler et al., 2006; Chen et al., 2008). It regulates Rac and PAK activity, proteins that are associated with regulation of the actin cytoskeleton dynamic, and participates in F-actin assembly (García Arguinzonis et al., 2002; Galler et al., 2006). Previous studies demonstrated that Ena/VASP-deficient endothelial cells are not able to reorganize stress fibers under physiological shear stress and that G-actin incorporation at barbed ends of stress fibers is diminished (Furman et al., 2007). Moreover, Ena/VASP deficiency is correlated with increased endothelial permeability (Furman et al., 2007). Another study showed that VASP is involved in stabilization of endothelial barrier through cAMP-dependent activation of Rac1 (Schlegel et al., 2008; Schlegel and Waschke, 2009). In fibroblasts and mesangial cells, VASP elimination enhances spreading (García Arguinzonis et al., 2002; Galler et al., 2006). Moreover, it was demonstrated that activation of Rac1/PAK pathway, an important part in the regulation of cell spreading, is significantly increased in VASP-deficient fibroblasts (García Arguinzonis et al., 2002). Interestingly, mutation of VASP phosphorylation sites (Ser239Ala, Ser157Ala, and Thr278Ala), VASP overexpression or VASP knock out mediate the formation of stable and thick actin stress fibers in fibroblasts and mesangial cells (Galler et al., 2006). The Galler et al. (2006) suggested a hypothesis that increased Rac/PAK pathway activity in VASP-deficient cells leads to stabilization of stress fibers.

The Rho GTPase Rac1 and its effector PAK are also expressed in podocytes, and their activation usually promotes podocyte injury and detachment as the result of foot process effacement (Robins et al., 2017). Despite the unknown role of VASP in regulating the signaling pathways in podocytes,

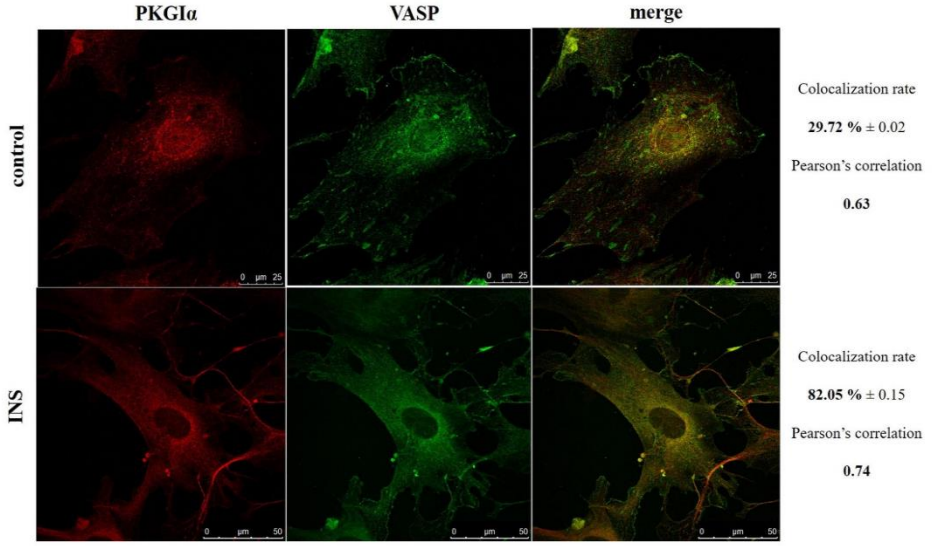

Figure 2 Subcellular distribution of protein kinase G Iα (PKGIα) and vasodilator-stimulated phosphoprotein (VASP) in cultured rat podocytes. The merged images show colocalization of PKGIα with VASP in differentiated rat podocytes. Cells seeded onto coverslips were incubated for 5 min in the absence or presence of insulin (INS, 300 nM). Podocytes were then immunostained with anti-PKGIα (1:15; Santa Cruz Biotechnology) and anti-VASP (1:30; Sigma Aldrich) antibodies. Quantitative analysis of protein colocalization was performed with LAS AF 3.3.0 software ($n = 3$, $P < 0.05$). The pixel intensities were quantified and the results are presented as Pearson's correlation coefficients and colocalization rates (%).

disturbances in VASP function may be involved in the regulation of rearrangement of the podocyte actin cytoskeleton through activation of the Rac1/PAK pathway.

Conclusions

Although the structure of VASP has been studied and described, the mechanism of action and the particular role of VASP in podocytes and kidney are poorly understood. VASP and its phosphorylation play an important role in regulating actin dynamics. A broad body of literature shows that VASP phosphorylation leads to disturbances in F-actin assembly and mediates regulation of other proteins associated with actin cytoskeleton rearrangement. In podocytes, earlier findings suggest that VASP phosphorylation contributes to increased motility and interacts with PKGIα after insulin stimulation. Understanding the role of VASP in cell signaling in podocytes may help with predicting the mechanisms responsible for the development of kidney diseases and identify VASP as a novel therapeutic target for treating these diseases.

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Conflict of interest

The authors declare that there are no conflict of interests.

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10. Pisemne oświadczenia autorów prac tworzących zbiór

10.1. Oświadczenia kandydata określające jego indywidualny wkład w powstanie każdej z prac wchodzących w skład zbioru.

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Oświadczenie

Oświadczam, że w pracy „Rachubik P, Szrejder M, Audzeyenka I, Rogacka D, Rychłowski M, Angielski S, Piwkowska A. The PKGIα/VASP pathway is involved in insulin- and high glucose-dependent regulation of albumin permeability in culture rat podocytes. J Biochem 2020, 2: mvaa059; doi: 10.1093/jb/mvaa059" mój udział polegał na: uczestniczeniu w sformuowaniu hipotezy badawczej oraz zaplanowaniu i wykonaniu doświadczeń, wykonaniu obliczeń oraz analizy statystycznej wyników, interpretacji wyników, przygotowaniu rycin, napisaniu manuskryptu a następnie dyskusji z recenzentami i ostatecznej redakcji manuskryptu.

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Oświadczenie

Oświadczam, że w pracy „Rachubik P i Piwkowska A. The role of vasodilator-stimulated phosphoprotein in podocyte functioning. *Cell Biol Int.* 43(10): 1092-1101" mój udział polegał na: uczestniczeniu w koncepcji manuskryptu, wykonaniu barwień immunofluorescencyjnych podocytów, przygotowaniu rycin, napisaniu manuskryptu a następnie dyskusji z recenzentami i edycji ostatecznej wersji dokumentu.

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Oświadczenie

Oświadczam, że w pracy „Rachubik P, Szrejder M, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A. The TRPC6-AMPK pathway is involved in insulin-dependent cytoskeleton reorganization and glucose uptake in cultured rat podocytes. Cell Physiol Biochem 2018, 51: 393-410" mój udział polegał na: wyprowadzaniu oraz hodowli pierwotnej linii komórek podocytarnych, wyciszaniu ekspresji genu kodującego TRPC6 za pomocą siRNA, oznaczaniu białek za pomocą metody Western blot, wykonaniu obliczeń oraz analizie statystycznej otrzymanych wyników w programie Sigma Plot 11.0, współudziale w napisaniu manuskryptu.

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Oświadczenie

Oświadczam, że w pracy „Rogacka D, Audzeyenka I, Rachubik P, Rychłowski M, Kasztan M, Jankowski M, Angielski S, Piwkowska A. Insulin increases filtration barrier permeability via TRPC6-dependent activation of PKGIα signaling pathways. Biochim. Biophys. Acta, Mol. Basis Dis 2017, 1863: 1312-1325" mój udział polegał na oznaczeniu przepuszczalności kłębuszków nerkowych dla albuminy *in vitro* i przygotowaniu opisu tej metody, wykonaniu obliczeń oraz analizie statystycznej otrzymanych wyników.

Patrycja Rachubik

10.2. 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, 4 listopada 2020

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im. M. Mossakowskiego, PAN
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Oświadczenie

Oświadczam, że w pracy „Rogacka D, Audzeyenka I, Rachubik P, Rychłowski M, Kasztan M, Jankowski M, Angielski S, Piwkowska A. Insulin increases filtration barrier permeability via TRPC6-dependent activation of PKGIα signaling pathways. Biochim. Biophys. Acta, Mol. Basis Dis 2017, 1863: 1312-1325" mój udział polegał na uczestniczeniu w koncepcji i sprawowaniu nadzoru merytorycznego nad wykonaniem projektu, interpretacją wyników oraz pisaniem i redakcją manuskryptu.

Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Piwkowska

Gdańsk, 4 listopada 2020

dr hab. Agnieszka Piwkowska, prof. IMDiK PAN
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02-106 Warszawa

Oświadczenie

Oświadczam, że w pracy „Rachubik P I Piwkowska A. The role of vasodilator-stimulated phosphoprotein in podocyte functioning. *Cell Biol Int,* 43(10): 1092-1101" mój udział polegał na uczestniczeniu w koncepcji i sprawowaniu nadzoru merytorycznego nad pisaniem i redakcją końcową manuskryptu.

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Piwkowska

Gdańsk, 5 listopada 2020

dr hab. Dorota Rogacka
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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Gdańsk, 5 listopada 2020

dr hab. Dorota Rogacka
Pracownia Molekularnej i Komórkowej Nefrologii
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Oświadczenie

Oświadczam, że w pracy „Rachubik P, Szrejder M, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A. The TRPC6-AMPK pathway is involved in insulin-dependent cytoskeleton reorganization and glucose uptake in cultured rat podocytes. Cell Physiol Biochem 2018, 51: 393-410" mój udział polegał na zaplanowaniu i wykonaniu doświadczeń, obliczeń i analizy statystycznej wyników związanych z insulinozależnym dokomórkowym transportem glukozy w podocytach oraz interpretacją uzyskanych wyników.

Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Gdańsk, 5 listopada 2020

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

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Gdańsk, 9 listopada 2020

Oświadczenie

Oświadczam, że w pracy „Rogacka D, Audzeyenka I, Rachubik P, Rychłowski M, Kasztan M, Jankowski M, Angielski S, Piwkowska A. Insulin increases filtration barrier permeability via TRPC6-dependent activation of PKGIα signaling pathways. Biochim. Biophys. Acta, Mol. Basis Dis 2017, 1863: 1312-1325" mój udział polegał na izolacji RNA ze szczurzych podocytów, przeprowadzeniu reakcji real-time PCR wraz z analizą i opracowaniem wyników eksperymentu oraz redagowaniu manuskryptu.

Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Irena Audzeyenka

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Gdańsk, 9 listopada 2020

Oświadczenie

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Irena Audzeyenka

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Gdańsk, 9 listopada 2020

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Irena Audzeyenka

Gdańsk, 6 listopada 2020

dr Michał Rychłowski

Zakład Biologii Molekularnej Wirusów

Międzyuczelniany Wydział Biotechnologii
Uniwersytetu Gdańskiego
i Gdańskiego Uniwersytetu Medycznego

ul. Abrahama 58

80-307 Gdańsk

Oświadczenie

Oświadczam, że w pracy „Rachubik P, Szrejder M, Rogacka D, Audzeyenka I, Rychłowski M, Angielski S, Piwkowska A. The TRPC6-AMPK pathway is involved in insulin-dependent cytoskeleton reorganization and glucose uptake in cultured rat podocytes. Cell Physiol Biochem 2018, 51: 393-410" mój udział polegał na obrazowaniu konfokalnym preparatów wykonanych metodą immunofluorescencyjną oraz na ich analizie.

Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Gdańsk, 6 listopada 2020

dr Michał Rychłowski

Zakład Biologii Molekularnej Wirusów

Międzyuczelniany Wydział Biotechnologii
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Oświadczenie

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Gdańsk, 6 listopada 2020

dr Michał Rychłowski

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Wyrażam zgodę na wykorzystanie publikacji w przewodzie doktorskim mgr Patrycji Rachubik.

Gdańsk, 24 listopada 2020

mgr Patrycja Rachubik
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Lp.	Imię i nazwisko	Wyszczególnienie
1.	mgr Maria Szrejder Pracownia Molekularnej i Komórkowej Nefrologii IMDiK PAN Katedra Biotechnologii Molekularnej Wydział Chemii Uniwersytet Gdański ul. Wita Stwosza 63 80-308 Gdańsk	-formatowaniu, omówieniu oraz końcowej akceptacji manuskryptu;
2.	prof. dr hab. Stefan Angielski Pracownia Molekularnej i Komórkowej Nefrologii IMDiK PAN Katedra Biotechnologii Molekularnej Wydział Chemii Uniwersytet Gdański ul. Wita Stwosza 63 80-308 Gdańsk	- sprawowaniu opieki merytorycznej oraz końcowej akceptacji manuskryptu;

Patrycja Rachubik

Gdańsk, 24 listopada 2020

mgr Patrycja Rachubik
Pracownia Molekularnej i Komórkowej Nefrologii
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Patrycja Rachubik

Gdańsk, 24 listopada 2020

mgr Patrycja Rachubik
Pracownia Molekularnej i Komórkowej Nefrologii
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Lp.	Imię i nazwisko	Wyszczególnienie
1.	dr Małgorzata Kasztan Department of Medicine University of Alabama at Birmingham 1720 University Blvd, Birmingham, AL 35294, USA	- oznaczaniu przepuszczalności izolowanych kłębuszków nerkowych dla albuminy;
2.	prof. dr hab. Maciej Jankowski Zakład Chemii Klinicznej Gdański Uniwersytet Medyczny ul. Dębinki 7 80-211 Gdańsk	- końcowej akceptacji manuskryptu;
3.	prof. dr hab. Stefan Angielski Pracownia Molekularnej i Komórkowej Nefrologii IMDiK PAN Katedra Biotechnologii Molekularnej Wydział Chemii Uniwersytet Gdański ul. Wita Stwosza 63 80-308 Gdańsk	- sprawowaniu opieki merytorycznej oraz końcowej akceptacji manuskryptu;

Patrycja Rachubik