lek. Krzysztof Bartkowiak

Rola sygnalizacji VEGF/VEGFR-2 w regulacji odpowiedzi angiogennej śródbłonka i przepuszczalności mikronaczyń w miokardium w mysim modelu zespołu metabolicznego

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

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Spis treści

1. Wykaz stosowanych skrótów

AKT – inaczej PKB – protein kinase B (kinaza białkowa B)

BMI – body mass index (wskaźnik masy ciała)

CALN – calcineurin (kalcyneuryna)

DAG – diglyceride (digliceryd)

eNOS – endothelial nitric synthase (śródbłonkowa syntaza tlenku azotu)

ERK1 – extracellular signal-regulated kinase 1

(kinaza regulowana sygnałem zewnątrzkomórkowym 1)

ERK1/2 – extracelluar signal-regulated kinase 2

(kinaza regulowana sygnałem zewnątrzkomórkowym 2)

FAK – focal adhesion kinase (kinaza płytek przylegania)

FGF – fibroblast growth factor (czynnik wzrostu fibroblastów)

FOXO1 – forkead box protein O1 (czynnik transkrypcyjny FOXO1)

FOXO3a – forkead box protein O3a (czynnik transkrypcyjny FOXO3a)

IDF – Internation Diabetes Federation (Międzynarodowa Federacja Diabetologiczna)

IP3 – inositol trisphosphate (trifosforan inozytolu)

Ki-67 – marker of proliferation Kiel 67 (marker proliferacji komórkowej Kiel 67)

LEPR – leptin recetor (receptor dla leptyny)

MAP – mitogen-activated protein kinase (kinaza aktywowana mitogenem)

MetS – metabolic syndrome (zespół metaboliczny)

mRNA – messenger RNA (matrycowe RNA)

mTOR – mammalian target of rapamycin (ssaczy cel rapamycyny)

NFAT – nuclear factor od activated T-cells

(czynnik jądrowy aktywowanych limfocytów T)

NO – nitric oxide (tlenek azotu)

NRP-1 – neuropilin 1 (neuropilina 1)

p38 – protein 38 mitogen-activated protein kinase (kinaza aktywowana mitogenem p38)

PI3K – phosphoinositide 3-kinase (kinaza 3-fosfatydyloinozytolu)

PIP3 – phosphatidylinositol (3,4,5) – trisphosphate

(5'-fosforan (3,4,5)-trifosforanu fosfatydyloinozytolu)

PKC – protein kinase C (kinaza białkowa C)

PLCϒ – phospholipase C (fosfolipaza C)

PTEN – phosphatase and tensin homog deleted on chromosome ten
(homolog fosfatazy i tensyny, ulegający delecji w chromosomie 10)
PXN – paxillin (paksylina)
RAF-1 – proto-oncogene serine/threonine-protein kinase c-RAF
(specyficzna kinaza serynowo-treoninowa)
SRC – proto-oncogene tyrosine protein kinase SRC (specyficzna kinaza tyrozynowa)
STAT – signal transducer and activator of transcription
(przekaźnik sygnału i aktywator transkrypcji)
VEGF – vascular endothelial growth factor (naczyniowo-śródbłonkowy czynnik wzrostu)
VEGF-A - vascular endothelial growth factor A
(naczyniowo-śródbłonkowy czynnik wzrostu A)
VEGF-B – vascular endothelial growth factor B
(naczyniowo-śródbłonkowy czynnik wzrostu B)
VEGF-C – vascular endothelial growth factor C
(naczyniowo-śródbłonkowy czynnik wzrostu C)
VEGF-D – vascular endothelial growth factor D
(naczyniowo-śródbłonkowy czynnik wzrostu D)
VEGFR – vascular endothelial growth factor receptor
(receptor dla naczyniowo-śródbłonkowego czynnika wzrostu)
VEGFR-1 – vascular endothelial growth factor receptor type 1
(receptor dla naczyniowo-śródbłonkowego czynnika wzrostu typ 1)
VEGFR-2 – vascular endothelial growth factor receptor type 2
(receptor dla naczyniowo-śródbłonkowego czynnika wzrostu typ 2)
VEGFR-3 – vascular endothelial growth factor receptor type 3
(receptor dla naczyniowo-śródbłonkowego czynnika wzrostu typ 3)
VE-kadheryna – vascular endothelial kadherin (kadheryna śródbłonka naczyniowego)
WHO – World Health Organization (Światowa Organizacja Zdrowia)
WOBASZ - Wieloośrodkowe Ogólnopolskie Badanie Stanu Zdrowia Ludności
ZDF – Zucker Diabetic Rat (szczurzy model cukrzycy)

2. Streszczenie w języku polskim

Zespół metaboliczny (MetS) definiowany jest jako zespół współistniejących schorzeń, takich jak otyłość, zaburzenia gospodarki węglowodanowej oraz lipidowej, insulinooporność oraz podwyższone wartości ciśnienia tętniczego. MetS stanowi istotny problem chorobowy, gdyż dotyka co trzeciej kobiety oraz blisko 40% mężczyzn w Polsce. W tej grupie pacjentów szczególnie zwiększone jest ryzyko zdarzeń sercowo-naczyniowych oraz obserwowany jest rozwój niewydolności serca z zachowaną frakcją wyrzutową. W patogenezie powikłań sercowo-naczyniowych w MetS ważną rolę odgrywa postępująca dysfunkcja komórek śródbłonka naczyniowego w sercu, a co za tym idzie, mikrokrążenia. Objawy MetS, takie jak hiperglikemia, hipercholesterolemia, czy nadciśnienie, mają negatywny wpływ na metabolizm komórek śródbłonka poprzez indukcję stanu zapalnego, zaburzenia w gospodarce wolnymi rodnikami, czy deregulację produkcji NO. Powyższe zmiany prowadzą do sztywnienia ściany naczyń, zwiększonej przepuszczalności bariery naczyniowej i upośledzenia procesów angiogenezy. Rezultatem jest upośledzenie krążenia, prowadzące do obrzęku, włóknienia i niewydolności serca.

Celem prac było określenie roli, jaką odgrywa sygnalizacja VEGF/VEGFR-2 w odpowiedzi angiogennej śródbłonka oraz regulacji przepuszczalności mikronaczyń miokardium w MetS na modelu myszy db/db, które wykazują cechy zespołu metabolicznego.

Jako model do doświadczeń wykorzystano myszy db/db, które na skutek spontanicznej mutacji nie posiadają funkcjonalnego receptora dla leptyny. Zwierzęta te nie kontrolują apetytu, a co za tym idzie rozwijają otyłość, cukrzycę i hipercholesterolemię. Myszy karmione były standardową paszą i w 21. tygodniu życia pobrano od nich serce oraz aortę. Aby potwierdzić występowanie cech zespołu metabolicznego u zwierząt doświadczalnych, w odstępach tygodniowych były one poddawane ważeniu i mierzono poziom glikemii. Gęstość naczyń krwionośnych, receptora dla VEGF i VE-kadheryny w miokardium oceniana była z wykorzystaniem barwień immunohistochemicznych i mikroskopii konfokalnej. Ultrastrukturę komórek śródbłonka w miokardium, ze szczególnym uwzględnieniem połączeń między komórkami śródbłonka i liczebności pęcherzyków transcytotycznych oceniano w transmisyjnym mikroskopie elektronowym. Następnie z miokardium na drodze enzymatycznej i przy zastosowaniu techniki sortowania na kolumnie magnetycznej wyizolowano komórki śródbłonka naczyniowego i przeprowadzono analizę ekspresji wybranych mRNA

(między innymi VEGF-A, VEGF-B, VEGFR-2, AKT, SRC, ERK1, VE-kadheryna) używając techniki Real-Time PCR. Ponadto wykonano test stymulacji krążków aortalnych w celu oceny potencjału angiogennego śródbłonka.

W badaniach wykazano, że mysz db/db karmiona paszą standardową szybko przybiera na wadze, a pomiary stężenia glukozy wykazały hiperglikemię, jednakże różnice pomiędzy osobnikami w obrębie jednej grupy były znaczące. W miokardium myszy db/db obserwowano zmniejszoną liczba mikronaczyń krwionośnych w stosunku do zwierząt kontrolnych. Ponadto w analizie ultrastruktury naczyń mikrokrążenia w koniuszkowej części serca zaobserwowano zwiększenie przestrzeni pomiędzy komórkami śródbłonka naczyniowego oraz spadek intensywności transportu pęcherzykowego, co może wskazywać na rozszczelnienie bariery naczyniowej. Wykazano, że ekspresja mRNA dla VEGF-A i VEGFR-2 jest zwiększona w izolowanych komórkach śródbłonka naczyniowego myszy db/db, natomiast analiza immunohistochemiczna gęstości ekspresji VEGFR-2 w komórkach śródbłonka wykazała, że dochodzi do jej zmniejszenia w całym sercu, w szczególności w obszarze przegrody międzykomorowej. Wykazano, że w izolowanych komórkach śródbłonka naczyń serca dochodzi do zwiększenia ekspresji mRNA dla kluczowych białek zaangażowanych w przekazywanie sygnału wewnątrzkomórkowego regulującego angiogenezę (szlak kinazy AKT i MAP) oraz przepuszczalność bariery naczyniowej (szlak kinazy SRC); kiedy analizę przeprowadzono na fragmencie tkanki miokardium różnic w ekspresji nie obserwowano. Jednocześnie wykazano na drodze barwienia immunohistochemicznego, że w obszarze lewej komory dochodzi do spadku ekspresji VE-kadheryny w komórkach śródbłonka serca myszy db/db. Test krążków aortalnych wykazał, że komórki śródbłonka myszy db/db mają mniejszy potencjał angiogenny, gdyż po pobudzeniu przez VEGF-A wytwarzają mniej naczyniopodobnych struktur niż aorty myszy z grupy kontrolnej.

Otrzymane wyniki mogą sugerować, że w MetS dochodzi do istotnego upośledzenia odpowiedzi angiogennej komórek śródbłonka serca, zależnej od szlaku sygnałowego VEGF-VEGFR-2. Ponadto analiza izolowanych komórek śródbłonka pozwala przybliżyć mechanizmy, które regulują proces angiogenezy i przepuszczalności naczyń krwionośnych u myszy db/db, co nie jest możliwe, kiedy podobne badania prowadzi się na fragmencie miokardium. Uzyskane wyniki mogą być wstępem do dalszych badań nad dysfunkcją mikrokrążenia w MetS, a co za tym idzie w przyszłości mogą się przyczynić do opracowania nowych metod diagnostycznych i terapeutycznych niewydolności serca w zespole metabolicznym.

3. Streszczenie w języku angielskim (wraz z angielską wersją tytułu rozprawy)

The role of VEGF/VEGFR-2 signaling in the regulation of endothelial angiogenic response and microvascular permeability in the myocardium in a mouse model of metabolic syndrome

Metabolic syndrome (MetS) is defined as a set of coexisting abnormalities, such as obesity, carbohydrate and lipid metabolism disorders, insulin resistance and elevated blood pressure. MetS is a significant disease problem as it affects every third woman and nearly 40% of men in Poland. In this group of patients the risk of cardiovascular events is particularly increased and the development of heart failure with preserved ejection fraction is observed. In the pathogenesis of cardiovascular complications in MetS, an important role is played by the progressive dysfunction of vascular endothelial cells in the heart and, consequently, of the microcirculation. Symptoms of MetS, such as hyperglycemia, hypercholesterolemia, or hypertension, have a negative impact on the metabolism of endothelial cells by inducing inflammation, disturbances in the metabolism of free radicals, and deregulation of NO production.The above changes lead to stiffening of the vascular wall, increased permeability of the vascular barrier and impaired angiogenesis processes. The result is impaired circulation, leading to edema, fibrosis, and heart failure.

The aim of the study was to determine the role of VEGF/VEGFR-2 signaling in the endothelial angiogenic response and the regulation of myocardial microvascular permeability in MetS in the db/db mouse model, which exhibits features of the metabolic syndrome.

The db/db mouse was used as a model for the experiments, which, due to a spontaneous mutation, lacks a functional leptin receptor. These animals do not control their appetite and therefore develop obesity, diabetes and hypercholesterolemia. The mice were fed standard feed and at 21 weeks of age their heart and aorta were collected. To confirm the presence of features of the metabolic syndrome in experimental animals, they were weighed at weekly intervals and their glycemia levels were measured. The density of blood vessels, VEGF receptor and VE-cadherin in the myocardium was assessed using immunohistochemical staining and confocal microscopy. The ultrastructure of endothelial cells in the myocardium, with particular emphasis on junctions between endothelial cells and the number of transcytotic vesicles, was assessed using a transmission

electron microscope. Then, vascular endothelial cells were isolated from the myocardium using an enzymatic method and a magnetic column sorting technique, and an analysis of the expression of selected mRNAs (including VEGF-A, VEGF-B, VEGFR-2, AKT, SRC, ERK1, VE-cadherin) was performed using the Real-Time PCR. Additionally, an aortic ring assay was performed to assess the angiogenic potential of the endothelium.

The study showed that db/db mice fed standard diet quickly gained weight, and glucose measurements showed hyperglycemia, but the differences between individuals within one group were significant. A reduced number of blood microvessels was observed in the myocardium of db/db mice compared to control animals. Moreover, the ultrastructure analysis showed an increase in the space between vascular endothelial cells and a decrease in the intensity of vesicular transport in the apical region of the heart, which may indicate a weakening of the vascular barrier. VEGF-A and VEGFR-2 mRNA expression was shown to be increased in isolated vascular endothelial cells of db/db mice, while immunohistochemical analysis of VEGFR-2 expression density in endothelial cells showed that it was decreased throughout the heart, especially in the interventricular septum area. It has been shown that in isolated cardiac endothelial cells there is an increase in the expression of mRNA for key proteins involved in the transmission of intracellular signals regulating angiogenesis (AKT and MAP kinase pathway) and vascular barrier permeability (SRC kinase pathway); when the analysis was performed on a fragment of myocardial tissue, differences in expression were not observed. At the same time, immunohistochemical staining showed that there is a decrease in VE-cadherin expression in the cardiac endothelial cells of db/db mice in the left ventricular area. The aortic ring assay showed that the endothelial cells of db/db mice have a lower angiogenic potential because they produce fewer vascular-like structures when stimulated by VEGF-A than the aortas of control mice.

The obtained results may suggest that in MetS there is a significant impairment of the angiogenic response of cardiac endothelial cells, which is dependent on the VEGF-VEGFR-2 signaling pathway. Moreover, the analysis of isolated endothelial cells allows us to elucidate the mechanisms that regulate the process of angiogenesis and blood vessel permeability in db/db mice, which is not possible when similar studies are carried out on a fragment of the myocardium. The obtained results may be an introduction to further research on microcirculation dysfunction in MetS, and thus in the future they may contribute to the development of new diagnostic and therapeutic methods for heart failure in MetS.

4. Wprowadzenie

4.1 Zespół metaboliczny

Zespół metaboliczny definiowany jest jako zespół współistniejących schorzeń, takich jak otyłość, zaburzenia gospodarki węglowodanowej i lipidowej, insulinooporność oraz podwyższone wartości ciśnienia tętniczego. Zgodnie z wytycznymi WHO głównym kryterium rozpoznania MetS jest otyłość oraz występowanie dwóch z trzech: nadciśnienia, hiperglikemii lub hipercholesterolemii. Badania nad MetS wydają się szczególnie interesujące, gdyż rozpowszechnienie tej jednostki chorobowej dynamicznie się zwiększa, szczególnie w krajach rozwiniętych i dotyczy mniej więcej 1/3 całej populacji [1]. W Polsce częstość występowania MetS była oceniana w badaniach WOBASZ. Jak wskazują wyniki danych, zbieranych w latach 2013-2014, problem ten dotyczy 32,8% kobiet oraz 39% mężczyzn. Istotniejsze natomiast jest to, że na przestrzeni około 10 lat w porównaniu z danymi z badania WOBASZ I zachorowalność wzrosła o 3,3% u kobiet oraz o 8,8% u mężczyzn [2].

Definicja MetS zmieniała się na przestrzeni lat. Szczególną uwagę zwracano na otyłość, zwłaszcza typu brzusznego, obecność nadciśnienia tętniczego, podwyższoną glikemię czy zaburzenia lipidowe. Poszczególne progi, które były brane jako punkty odcięcia różnią się w zależności od kryteriów przyjętych przez WHO czy IDF [3]. W Polsce obowiązują ustalenia z 2022 roku, wedle których do rozpoznania MetS kwalifikuje obwód talii powyżej 88 cm u kobiet oraz powyżej 102 cm u mężczyzn lub BMI $\geq$30 kg/m^2, oraz obecności dwóch z trzech zaburzeń, takich jak cukrzyca lub stan przedcukrzycowy, podwyższone stężenie cholesteroli oraz wysokie ciśnienie tętnicze [4].

4.2. Modele zwierzęce MetS

W badaniach nad MetS stosuje się modele zwierzęce trzech rodzajów, w których objawy zespołu są wynikiem mutacji genetycznej, karmienia specjalnie dobraną paszą lub podawania leków, mających indukować zaburzenia metaboliczne. W modelu indukowanym dietą najczęściej stosowana są mieszanki paszowe z dodatkiem węglowodanów, jakich jak fruktoza czy sacharoza, lub diety wysokotłuszczowe, lub mieszanki powyższych. Wśród leków stosuje się glikokortykosteroidy lub leki przeciwpsychotyczne. Obie powyższe metody indukcji MetS są czasochłonne, dlatego

często stosowane są również modele oparte na mutacjach genetycznych, takie jak myszy db/db czy szczury ZDF [5].

4.3. Myszy db/db z mutacją spontaniczną w receptorze dla leptyny

Myszy db/db wykazują spontaniczną mutację w receptorze dla leptyny, co powoduje jego inaktywację. Centralne zaburzenia sygnalizacji leptyna – LEPR rozregulowują ośrodek sytości w mózgu, w wyniku czego dochodzi do pojawienia się u tych zwierząt otyłości, zaburzeń gospodarki węglowodanowej i hipercholesterolemii. Ponadto zmiany metaboliczne u tych zwierząt wywołują także zmiany morfologiczne i funkcjonalne w mięśniu sercowym, prowadząc do postępującej niewydolności tego narządu [6].

Leptyna odgrywa kluczową rolę w homeostazie energetycznej przez wpływ na ośrodki głodu i sytości w podwzgórzu. Ponadto ta adipokina, która jest głównie produkowana w tkance tłuszczowej żółtej, wywiera liczne efekty obwodowe, gdyż wiele typów komórek posiada receptory dla tego białka. Udział obwodowej sygnalizacji leptyna - LEPR opisano, między innymi, w układzie rozrodczym, odpornościowym i sercowo-naczyniowym [7]. U pacjentów z MetS obserwowane jest podwyższone stężenie leptyny w surowicy. Powoduje to oporność na stymulację leptyną nie tylko receptorów zlokalizowanych w podwzgórzu, ale także innych komórkach organizmu. Oporność na leptynę zwiększa predyspozycję do wystąpienia otyłości, co z kolei dalej podnosi poziom tego hormonu we krwi i prowadzi do pogłębienia istniejącej oporności na leptynę [8]. Ponadto są również dane, które sugerują, że do niewrażliwości receptorów na leptynę dochodzi w kardiomiocytach [9]. U pacjentów z otyłością zmiany w szlakach sygnałowych zależnych od leptyny zachodzą również na poziomie komórkowych. W śródbłonku, w stanie ciągłej stymulacji przez podwyższone stężenie leptyny, zmniejsza się gęstość receptorów LEPR, co powoduje upośledzoną odpowiedź tych komórek na stymulację leptyną. Nieprawidłowe działanie leptyny skutkuje intensywniejszym rozwojem przewlekłego stanu zapalnego oraz miażdżycy, jednak mechanizmy molekularne, które regulują te procesy nie zostały dokładnie opisane, szczególnie w naczyniach mikrokrążenia serca [10].

4.4. Śródbłonek naczyniowy w MetS

Diagnozowanie i monitorowanie pacjentów z MetS ma istotne znaczenie, chociażby z tego powodu, że w tej grupie szczególnie wzrasta ryzyko zdarzeń sercowo-naczyniowych i zgonu z powodu niewydolności serca [11]. Wdrożenie odpowiednich modyfikacji stylu życia oraz adekwatnego leczenia farmakologicznego istotnie to ryzyko zmniejsza [4]. Współistnienie otyłości, cukrzycy oraz zaburzeń lipidowych powoduje uszkodzenie wielu narządów, w szczególności serca [12, 13]. W MetS dochodzi między innymi do przerostu kardiomiocytów, zwiększonej produkcji wolnych rodników, pogorszenia funkcjonowania mikrokrążenia wieńcowego, a w konsekwencji obrzęku śródtkankowego. Wszystkie te zjawiska przyczyniają się do indukcji włóknienia, a w konsekwencji do zwiększenia sztywności ścian serca [14]. Ponadto uszkodzeniu ulegają również komórki śródbłonka naczyniowego. W MetS obserwowane jest upośledzenie funkcji śródbłonka spowodowane wzrostem stężenia wolnych rodników, wzrostem ekspresji cząsteczek adhezyjnych związanych ze stanem zapalnym i spadkiem stężenia NO. W mikrokrążeniu obserwuje się również szereg zmian morfologicznych, takich jak pogrubienie błony podstawnej oraz spadek gęstości kapilar [15]. W konsekwencji wyżej opisanych zmian dochodzi do zmniejszenia zaopatrzenia komórek serca w składniki odżywcze oraz tlen, a także obserwowany jest naciek zapalny oraz zwiększona liczba makrofagów [16, 17]. Prowadzi to do rozwoju charakterystycznej dla zespołu metabolicznego rodzaju niewydolności serca z zachowaną frakcją wyrzutową lewej komory [18].

4.5. Regulacja angiogenezy - oś VEGF-VEGFR

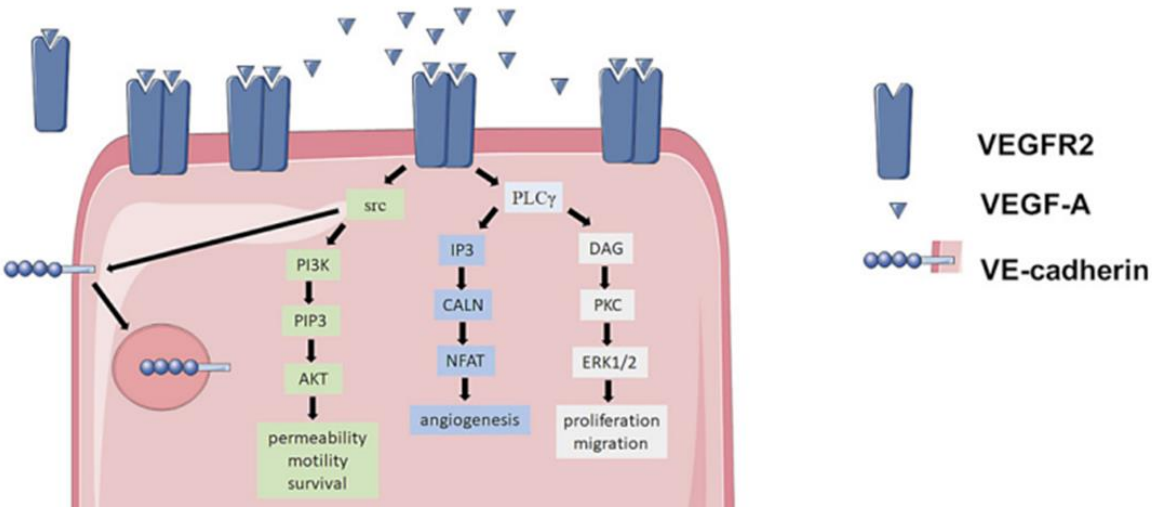
W procesie powstawania naczyń krwionośnych kluczową rolę odgrywają czynniki proangiogenne jakie jak VEGF czy FGF oraz receptory dla tych czynników wzrostu. Ponadto istotne są również inne białka kostymulujące np. neuropilina Rodzina VEGF składa się z pięciu cząsteczek – VEGF-A, VEGF-B, VEGF-C, VEGF-D oraz łożyskowego czynnika wzrostu, które wiązać się mogą z trzema receptorami – VEGFR-1, VEGFR-2 oraz VEGFR-3. Zarówno ligandy, jak ich receptory, mogą wywierać działanie pro- i antyangiogenne, dlatego też regulacja angiogenezy wymaga ścisłego ich współdziałania [19]. Aktywacja tych receptorów powoduje pobudzenie wewnątrzkomórkowych przekaźników. Wśród nich za regulowanie odpowiedzi angiogennej, poprzez stymulowanie podziałów komórkowych, migrację komórek

czy tworzenie nowych wypustek odpowiada szlak zależny od kinazy AKT oraz MAP. Natomiast zmiany w połączeniach komórkowych, a poprzez to, w ich przepuszczalności, są głównie zależne od aktywacji kinazy SRC i AKT [20].

VEGFR-1 najsilniej wiąże VEGF-A, aczkolwiek jego aktywacja jest słabsza w porównaniu do VEGFR-2, co sprawia, że biologiczny efekt, wywierany przez VEGFR-A jest słabszy [21]. Kaskada inicjowana przez interakcję VEGF-A/VEGFR-1 nie jest w stanie zainicjować angiogenezy, ale jest kluczowa dla dojrzewania nowo powstających naczyń i ich stabilizacji [22, 23]. Jako produkt alternatywnego składania, powstaje również skrócona, rozpuszczalna forma tego receptora, której zadaniem jest „wyłapywanie" VEGF-A i zmniejszanie jego biodostępności [24, 25].

VEGFR-2 jest kluczowy w inicjacji wczesnych etapów angiogenezy i waskulogenezy [26]. Receptor ten wykorzystuje aktywność koreceptorów – neuropilin, a szczególnie neuropiliny 1. Kompleks VEGFR-2/NRP-1 ma sześciokrotnie większą aktywność niż sam VEGFR-2 [27]. VEGFR-2 może również tworzyć heterodimery z VEGFR-1, co będzie modulowało odpowiedź na pobudzenie przez VEGF-A [28]. Podobnie jak VEGFR-1, również VEGFR-2 może występować w skróconej, rozpuszczalnej formie [29].

VEGFR-3 jest receptorem zaangażowanym we wzrost naczyń limfatycznych, chociaż ma również udział w angiogenezie naczyń krwionośnych [30, 31]. Może on tworzyć heterodimery z VEGFR-2, co reguluje początkowe etapy angiogenezy [32].
Główne szlaki regulujące angiogenezę i przepuszczalność naczyń zostały przedstawione na Rycinie 1.



Rycina 1. Główne szlaki zależne od VEGF i VEGFR, regulujące angiogenezę i przepuszczalność naczyń. Za J Vasc Res 2024;61:151–159, DOI: 10.1159/000538361.

4.6. Kaskady sygnałowe związane z receptorem VEGFR-2

Pobudzenie receptora VEGFR-2 inicjuje cytoplazmatyczną kaskadę sygnałową. Najlepiej poznanymi szlakami, transmitującymi sygnał w tym układzie są: (1) szlak zależny od fosfolipazy C/kinazy ERK1/2, (2) ścieżka kinaza PI3K/kinaza B/kinaza mTOR oraz (3) szlak kinazy SRC. Dodatkowo aktywacja VEGFR-2 może pobudzać kinazę p38 (MAPK) oraz białka STAT [20].

W procesie angiogenezy postnatalnej kluczową rolę odgrywa ścieżka związana z kinazą ERK1/2 [33-35]. Z kolei szlak zależny od kinazy B (AKT) reguluje przeżywalność komórek śródbłonka i ich zdolność do ruchu [36]. Ścieżka zależna od kinazy SRC może także bezpośrednio oddziaływać z VE-kadherynami, powodując ich internalizację lub degradację, co zwiększa przepuszczalność ściany naczynia [28].

4.7. Zaburzenia angiogenezy w MetS

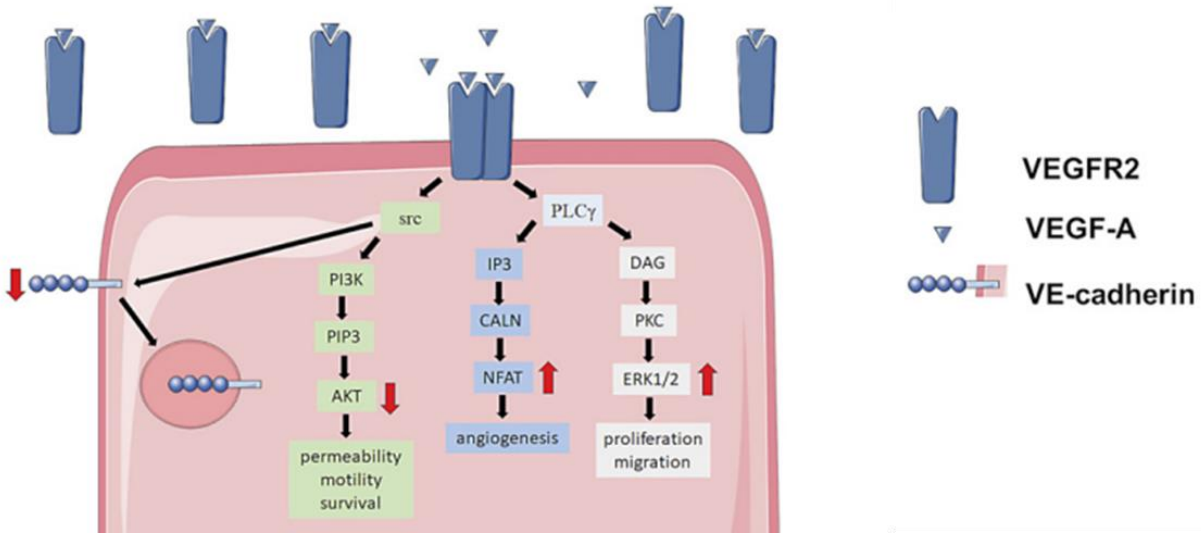
Nieprawidłowe tworzenie nowych naczyń krwionośnych z wcześniej istniejących struktur w MetS jest opisywane w literaturze, natomiast mechanizmy molekularne, które warunkują upośledzoną angiogenezę nie są dokładnie poznane [37, 38]. U pacjentów chorujących na MetS obserwuje się istotne zwiększenie stężenia VEGF w surowicy krwi, a co więcej, stężenie to pozytywnie koreluje z BMI, otyłością brzuszną i ogólnym poziomem tkanki tłuszczowej [39, 40]. Większe stężenie VEGF obserwowane jest również u pacjentów, u których już wystąpiły komplikacje naczyniowe lub obserwuje się nadciśnienie [41, 42]. Mimo tego tworzenie się krążenia obocznego u pacjentów z MetS po zawale jest upośledzone [43]. Może to świadczyć o zaburzonej sygnalizacji w kaskadzie VEGF-VEGFR.

Pomimo wyższego stężenia ligandów dla VEGFR w surowicy chorych na MetS nie obserwuje się u nich zwiększonej angiogenezy, a raczej zanik naczyń krwionośnych [17, 44]. Jedną z hipotez, która mogłaby tłumaczyć ten fenomen, jest spadek ekspresji receptorów dla VEGF, idący w parze ze zwiększonym stężeniem rozpuszczalnych form tych receptorów, które działają jak receptory wabikowe, zmniejszając biodostępność VEGF [29]. Hiperlipidemia może też powodować podwyższenie ekspresji receptora VEGFR-1, który negatywnie reguluje angiogenezę i może zwiększać przepuszczalność naczyń, co obserwowano na modelach danio pręgowanego [45].

4.8. Zaburzenia kaskad sygnałowych związanych z pobudzeniem VEGFR-2 w MetS.

Informacje dotyczące zaburzeń w ekspresji i funkcjonowaniu cząsteczek przekazujących sygnał z receptora VEGFR w MetS są bardzo skąpe. U myszy db/db i szczurów ZDF obserwuje się aktywację ERK1/2 w całym miokardium, natomiast aktywność kinazy B/Akt istotnie spada, jednakże w badaniach tych nie uwzględniono izolowanych komórek śródbłonka [46, 47]. Natomiast w modelu świńskim takich zmian nie obserwowano [48]. Obniżona aktywność kinazy B/AKT obserwowana jest również u zwierząt, u których MetS jest indukowany dietą [49, 50].

Aktywacja osi VEGF/VEGFR-2/kinaza SRC może prowadzić do fosforylacji VE-kadheryny, co powoduje rozpad połączeń międzykomórkowych i zwiększa przepuszczalność naczyń krwionośnych [51]. Na modelu świńskim, w którym MetS był indukowany dietą, obserwowano spadek ekspresji VE-kadheryny po zawale serca [52]. Zaburzenia szlaków sygnałowych regulujących angiogenezę i przepuszczalność naczyń w MetS zostały przedstawione na Rycinie 2.



Rycina 2. Zaburzenia głównych szlaków zależnych od VEGF i VEGFR regulujących angiogenezę i przepuszczalność naczyń w MetS. Za J Vasc Res 2024;61:151–159, DOI: 10.1159/000538361.

5. Założenia i cel pracy

Mając na uwadze rozpowszechnienie oraz rozwój chorób sercowo-naczyniowych u pacjentów z MetS wykonywane doświadczenia miały w głównej mierze stanowić próbę ustalenia mechanizmów w których dochodzi do pojawienia się niewydolności serca. Niewydolność serca jest efektem wieloczynnikowych zmian w morfologii i funkcjonowaniu tego narządu, ale zaburzenia, powodujące te zmiany są ciągłym przedmiotem badań. Poniższe prace skupiają się na jednym z ważniejszych aspektów tego zjawiska, czyli zaburzeniach w funkcjonowaniu śródbłonka naczyniowego w miokardium u pacjentów z MetS. Powszechnie wiadomo, że zaburzenia mikrokrążenia są wstępem do patologicznej przebudowy miokardium – zmniejszona liczba kapilar, wzrost ich przepuszczalności prowadzi do obrzęku śródtkankowego, co z kolei indukuje włóknienie i sztywnienie ściany serca, upośledzając jego kurczliwość.

Celem prac było określenie roli jaką odgrywa sygnalizacja VEGF/VEGFR-2 w odpowiedzi angiogennej śródbłonka oraz regulacji przepuszczalności mikronaczyń miokardium myszy db/db.

Zaplanowane doświadczenia miały za zadanie określić:

1. Jak zmienia się gęstość naczyń krwionośnych oraz ultrastruktura śródbłonka w sercach myszy z MetS?
2. Do jakich zmian dochodzi w ekspresji wybranych mRNA dla białek wchodzących w skład głównych kaskad sygnałowych, związanych z osią VEGF/VEGFR-2 w izolowanych komórkach śródbłonka naczyń serca myszy db/db?
3. Jak zmienia się gęstość receptora VEGFR-2 i VE-kadheryny w śródbłonku serc u myszy db/db?
4. Czy potencjalne zmiany ekspresji wybranych mRNA dla białek wchodzących w skład głównych kaskad sygnałowych, związanych z osią VEGF/VEGFR-2, będą miały wpływ na odpowiedź angiogenną śródbłonka myszy db/db?

6. Kopie opublikowanych prac

6.1. Praca: Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways

Wybrane do cyklu prace spójnie przedstawiają tematykę związaną z zaburzeniami, jakie są obserwowane w komórkach śródbłonka w sercu w warunkach MetS. Publikacja **Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways** jest przeglądem dostępnej literatury w zakresie zmian w szlaku sygnałowym zależnym od receptora VEGFR-2 w śródbłonku. Przedstawiono w nim podstawowe informacje odnośnie czynników wzrostu regulujących angiogenezę, a w szczególności VEGF-A. Ponadto opisano budowę oraz sposób aktywacji receptorów dla VEGF oraz receptorów drugiego rzędu, które pobudzane są przez ich stymulację. Główną część pracy poświęcono przedstawieniu zmian, które zostały dotychczas opisane w literaturze w kontekście szlaku sygnałowego zależnego od VEGF/VEGFR. Szczegółowo zostały opisane zaburzenia regulujące angiogenezę oraz przepuszczalność naczyń krwionośnych, co podsumowano na rycinie w publikacji.

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Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways

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Keywords
Metabolic syndrome · Heart failure · Angiogenesis · VEGF/VEGFR signaling · Vessel rarefaction

Abstract
Background: Elevated mortality rates in patients with metabolic syndrome (MetS) are partly due to adverse remodeling of multiple organs, which may lead to cardiovascular disease, nonalcoholic fatty liver disease, kidney failure, or other conditions. MetS symptoms, such as obesity, hypertension, hyperglycemia, dyslipidemia, associated with insulin and leptin resistance, are recognized as major cardiovascular risk factors that adversely affect the heart. ***Summary:*** Pathological cardiac remodeling is accompanied by endothelial cell dysfunction which may result in diminished coronary flow, dysregulated oxygen demand/supply balance, as well as vessel rarefaction. The reduced number of vessels and delayed or inhibited formation of collaterals after myocardial infarction in MetS heart may be due to unfavorable changes in endothelial cell metabolism but also to altered expression of vascular endothelial growth factor molecules, their receptors, and changes in signal transduction from the cell membrane, which severely affect angiogenesis. ***Key Messages:*** Given the established role of cardiac vessel endothelial cells in maintaining tissue homeostasis, defining the molecular background underlying vessel dysfunction associated with impaired angiogenesis is of great importance for future therapeutic purposes. Therefore, the aim of this paper was to present current information regarding vascular endothelial growth factor signaling in the myocardium of MetS individuals.

Introduction

The high mortality in patients with metabolic syndrome (MetS) is caused mainly by multiorgan dysfunction, as a result of increased cardiovascular risk factors, such as hypertension, obesity, and diabetes [1]. In combination, factors such as abdominal obesity, elevated

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blood pressure, an altered lipid profile, and abnormal carbohydrate metabolism play a crucial role in the pathogenesis of MetS [2]. MetS symptoms are mostly a result of poor lifestyle choices (smoking, unbalanced diet, and inadequate physical activity) and severely affect the EC metabolism due to oxidative stress and activation of pro-inflammatory pathways [3]. Structural remodeling of cardiac vessels including changes in extracellular matrix collagen-elastin ratio and vascular smooth muscle proliferation, but also impaired vasomotor function due to alterations in mineralocorticoid signaling, change mechanical properties of vessels, and affect blood flow. This, in turn, negatively affects oxygen and nutrient demand/supply balance [4, 5]. Impaired cardiac blood flow prompts adverse cardiac tissue remodeling which results in the development of one of the main MetS pathologies – heart failure with preserved ejection fraction [6, 7].

MetS microenvironment may also diminish the ability of ECs to respond to angiogenic stimuli [3]. In metabolically healthy animals, therapeutic proangiogenic agents, such as vascular endothelial growth factor (VEGF), stimulate collateral growth in cardiac muscle after induced heart infarction. These agents may reduce the size of the infarct and the risk of death. However, similar trials in patients with coronary disease have mostly failed [8, 9]. A diminished response of ECs to angiogenic stimuli is probably a result of their changed metabolism, reduced NO bioavailability, and elevated oxidative stress [8, 10]. Another cause of delayed or inhibited collateral growth in individuals with MetS may be a delayed phenotypic switch of vascular smooth muscle cells (VSMCs). During collateral vessel formation, VSMCs undergo the change from a contractile toward a proliferative phenotype, but to sustain blood vessel formation, they need to return to the contractile phenotype, which is inhibited in MetS [11]. Finally, MetS is associated with inflammation, which results in elevated expression of adhesion molecules on EC surfaces and inadequate infiltration by inflammatory cells, such as neutrophils, which in turn may affect collateral growth in MetS myocardium [12]. A poor angiogenic response and the accompanying EC death may, in turn, lead to a diminished density of micro-vessels and arterioles in cardiac muscle [4, 13–17].

The term "angiogenesis" describes the growth of new vessels via sprouting from preexisting ones. Under the influence of proangiogenic factors, ECs become motile and change their shape to form tip cells (elongated cells that become the spearhead of the sprout) and stalk cells (which establish the new vessel lumen and elongate the sprout via rapid proliferation). Finally, the new structure recruits mural cells, which stabilize it to enable blood flow (extensively reviewed in [18, 19]). Angiogenesis is regulated by several groups of different proteins. The most important ones include VEGFs, their receptors and coreceptors, as well as downstream cytoplasmic signaling molecules [20]. The VEGF family consists of five members – VEGF-A, VEGF-B, VEGF-C, VEGF-D, and the placental growth factor, which act via three basic receptors – VEGFR1, VEGFR2, and VEGFR3. Both the growth factors and receptors may occur in proangiogenic and anti-angiogenic isoforms, which enables subtle and precise regulation of angiogenesis (extensively reviewed in [21]). Not only VEGF molecules and their receptors play an important role in angiogenesis. The proper functioning of downstream signaling pathways is equally important [22]. Clinical observations show increased serum VEGF levels in patients with MetS [23]. Thus, levels of VEGF positively correlate with body mass index, obesity, and the amount of visceral adipose tissue [24, 25]. Serum VEGF elevation has been reported in type 2 diabetes mellitus, but only in patients with vascular complications [26]. On the other hand, VEGF levels are normal when blood glucose concentration is controlled [27]. Individuals with hypertension also have higher serum VEGF levels than normotensive individuals [28]. Despite higher VEGF levels, blood vessel formation and collateral vessel growth in patients with MetS are impaired after myocardial infarction [9]. This may be a result of EC dysfunction but also of the adversely affected VEGF signaling cascade.

Therefore, the aim of this paper was to present current information regarding VEGF signaling pathways, with a particular emphasis on VEGF-A and VEGFR2 in ECs in cardiac tissue during obesity and MetS. We searched through the PubMed database using key words such as "VEGFR1", "VEGFR2", "PLC-γ-ERK1/2", "SRC", "AKT", "MAPK", "VEGF signaling", "cardiac vessel", "heart failure", and "metabolic syndrome" and paid particular attention to literature reports from the last 5 years.

Vascular Endothelial Cell Growth Factors and Their Cellular Receptors

Molecules from the VEGF family have an affinity for transmembrane-spanning receptors that contain an intracellular domain with tyrosine kinase activity and seven

immunoglobulin-like extracellular domains. These domains are present in three isoforms of VEGF receptors – VEGFR1, VEGFR2, and VEGFR3 [20]. VEGFR1 (Fms-like TK-1, Flt-1) was described and characterized in the early 1990s [29]. This receptor binds with the greatest affinity to VEGF-A and regulates its biological effects. Although VEGFR1 strongly binds VEGF-A, it has a much weaker kinase activity when compared with that of VEGFR2. Therefore, the biological effect of VEGF-A via VEGFR1 activation is much weaker than that exerted via VEGFR2. VEGF-A/VEGFR1 signaling cascade is not crucial for angiogenesis initiation but for organization of new vessels maturation during later stages of this process [30, 31]. Since there are also different VEGF-A isoforms, VEGFR1 can form homodimers or heterodimers with VEGFR2, depending on the type of ligand. The heterodimers mainly limit VEGF-A-regulated activation of the receptor [32]. As a result of alternative splicing, a soluble, truncated form of VEGFR1 may be also formed [33]. Soluble VEGFR1 binds to VEGF-A and limits its biological availability, thus inhibiting VEGF function [33, 34]. Furthermore, by binding to low-density lipoproteins (LDL) and subsequent receptor phosphorylation, endocytosis, and degradation, VEGFR1 plays a crucial role in regulating the absorption of fatty acids by ECs [35].

The VEGFR2 subtype is another protein involved in VEGF/VEGFR signaling [36]. It plays an important role in early angiogenesis/vasculogenesis. Activation of the receptor occurs after binding a VEGF molecule, which causes VEGFR2 dimerization [37]. LDL may also activate this receptor [38]. In a most common pathway, the receptor is activated by VEGF-A but also by VEGF-C and VEGF-D [39]. Major coreceptors in VEGFR2 signaling are neuropilins (NRP). NRP-1 is mainly expressed in arterial ECs and binds VEGF-A, especially VEGF-A165. NRP-1 binds with VEGFR2 which results in an up to six times greater activation of this heterodimer when compared to activation without coreceptor. NRP-2 binds rather VEGF-C and enhances the effect of VEGFR3 activation [40]. VEGFR2 can form heterodimers with two other receptor subtypes – VEGFR1 and VEGFR2, which modulate VEGF signaling. VEGFR2 interacts with VEGFR1, which may sensitize ECs to VEGF, since VEGFR2 is phosphorylated by a VEGFR1 tyrosine kinase domain after heterodimerization. Finally, as a result of alternative splicing, also soluble form of VEGFR2 can be produced, which performs similar functions as soluble VEGFR1, including angiogenesis inhibition [20, 41].

VEGFR3, which is also known as Flt-4, is crucial for lymphatic vessel growth [42]. Moreover, new blood vessel formation is also dependent on VEGFR3 expression in blood endothelial cells [43]. Interaction of VEGFR3 with VEGFR2 results in the formation of a VEGFR2/VEGFR3 heterodimer, which regulates initial steps of angiogenesis [39]. Integrin-β1, syndecan-4, and neuropilin-2 (NRP-2) help VEGFR3 function normally [44].

Downstream Signaling in VEGF/VEGFR Pathways

VEGF receptor activation activates secondary messengers in cell cytoplasm. The most important molecules that are involved in VEGF signaling are phospholipase C γ (PLC-γ)/extracellular signal-regulated kinase (ERK1/2), phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, AKT)/mammalian target of rapamycin (mTOR), and SRC kinase pathways. In addition, activation of the VEGFR2 receptor triggers p38 mitogen-activated protein kinase (p38 MAPK) and signal transducer and activator of transcription (STAT) proteins [20].

The PLC-γ-ERK1/2 pathway plays a crucial role in postnatal angiogenesis. Dimerized VEGFR2 receptor undergoes phosphorylation at Y1173 in rodents and Y1175 in humans, which indirectly activates ERK1/2 and/or the nuclear factor of activated T cells (NFAT) [45]. First, PLC-γ is activated, which initiates production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). As a result of IP3 action, calcium ions are released from the endoplasmic reticulum and activate calmodulin and a serine-threonine kinase, e.g., calcineurin. These changes in cell signaling decrease VEGFR1 expression and enhance VEGFR2 proangiogenic function [20, 46]. DAG pathway activates protein kinase C (PKC) and then ERK1/2, which regulates ECs specification, proliferation, and migration by activating the E26 family of transcription factors (ETS) or causing the phosphorylation of histone type 7 deacetylases (HDAC7) [47].

The AKT serine-threonine kinase pathway regulates many processes in ECs. AKT is involved in the regulation of cell survival, motility, and vascular permeability [48]. Activation of this kinase requires binding through its PH domain with phosphatidylinositol-3,4,5-trisphosphate (PIP3), which is produced from phosphatidylinositol-4,5-diphosphate (PIP2) by a phosphoinositide 3-kinase (PI3K). The AKT-1 kinase, which works through the mTOR2 complex, is the most important molecule involved in the regulation of angiogenesis. VEGFR2 does not contain a domain, which can bind directly with PI3K, so activation of this kinase is mediated either by protein-tyrosine kinase SRC, VE-cadherin receptor domain, or AXL receptor tyrosine kinase (AXL) [20].

The SRC-dependent pathway is crucial for changes in cell shape, cell migration, and cell polarization, as well as regulating vascular permeability by intercellular junction modulation, e.g., via VE-cadherin phosphorylation in response to VEGF. SRC activation is determined by VEGFR2 phosphorylation at Y949 in rodents and Y951 in humans. Activated receptor binds T-cell-specific adapter (TSAd), which activates the SH3 domain of SRC. Moreover, shear stress accelerates SRC activation and controls the interaction between the cytoskeleton and cell adhesion proteins [49]. The SRC kinase also affects micro-vessel vessel permeability, phosphorylating VE-cadherin that results in the endocytosis of this protein and the destruction of adherent junctions, which disrupts vascular barrier [50].

VEGF signaling pathways can also involve p38 MAPK and STATs. MAPK induces angiogenesis, mostly by stimulating cell migration and EC survival. This pathway can also affect vascular wall permeability. Activation of p38 MAPK is dependent on NRP-1 costimulation of VEGFRs and the entry of calcium ions from the endoplasmic reticulum into cell cytoplasm. Ca2+ influx activates protein kinase 2β (PTK-2β), which – together with SRC – triggers the p38 MAPK cascade [20]. In addition, the STAT-1 and STAT-3 proteins are involved in cell cycle regulation, apoptosis, and activation of inflammatory molecules in the endothelium. STAT-3 also increases the density of vessels [51]. Main VEGF-VEGFR signaling pathways are summarized in Figure 1a.

Alterations in VEGFRs Expression in MetS

Despite a higher serum concentration of VEGFR ligands, individuals with MetS do not seem to have increased angiogenesis. One suspected mechanism behind this phenomenon is associated with altered VEGFR expression and increased levels of soluble VEGF receptors, which decrease VEGF bioavailability [41]. In the myocardium of db/db mice, known to develop MetS symptoms, a decrease in the expression of both VEGFR1 and VEGFR2 receptor subtypes is observed [52]. In the myocardium of obese and insulin-resistant rats, the expression of VEGFR1 and VEGFR2 mRNAs is significantly decreased. Furthermore, the expression of PECAM-1 protein, which is a molecular marker of endothelium, is also impaired in fa/fa diabetic rats, simultaneously with a diminished collateral vessel formation in these rats [53]. A reduction in microvascular and arteriolar density was also observed by Zeng et al. in db/db mouse hearts [14].

The presence of soluble VEGFR forms, which are responsible for limiting ligand bioavailability, is extremely important in VEGF signaling pathways. There is a significant increase in the amount of soluble form of VEGFR2 in the serum of patients with MetS symptoms, whereas there is no change in the concentration of soluble VEGFR1 [41]. On the other hand, hyperlipidemia, one of MetS symptoms, may increase the expression of VEGFR1, which has negative effects on angiogenesis and vessel integrity, as it was observed in zebrafish and mouse models [54]. Despite VEGF overexpression, there may be a poor angiogenic response of ECs in the heart in MetS, which is related to the altered VEGF signaling pathway. This poor angiogenic response can result from at least two mechanisms: (1) reduced VEGFR expression and/or (2) elevated concentration of sVERFR-2, which leads to limited VEGF-A availability.

VEGFR2 Downstream Signaling Cascade Alterations in MetS

PLC-γ/ERK1/2

There are limited data regarding the details of the PLC-γ-ERK1/2 pathway in the heart over the course of MetS. The existing data show that there are no major changes in the expression of ERK pathway-dependent kinases in the cardiomyocytes of db/db mice with MetS [55]. On the other hand, an increased activation of ERK in the whole myocardium observed in db/db mice may suggest that this pathway may be affected in ECs or other non-cardiomyocyte cardiac cells [56]. In the hearts of Zucker diabetic fatty (ZDF) rats, ERK kinase phosphorylation is increased, indicating ERK kinase activation [57]. These observations suggest that ECs can be a potential source of the observed changes in ERK signaling in the myocardium, since ECs are more numerous than either fibroblasts or macrophages. Moreover, an increased ERK1/2 phosphorylation (and thus ERK1/2 activation) has been observed in the aorta of diet-induced obese and diabetic rats. Of interest, physical exercise in these animals reduced ERK1/2 activation [58]. Conversely, ERK phosphorylation was not detected in a porcine model of MetS [59]. Changes in lipid metabolism, which also occur in MetS, can impact ERK signaling. The addition of the very low-density lipoproteins (VLDLs) isolated from MetS individuals and added to atrial myocyte cell lines cultured in vitro resulted in a diminished dephosphorylating activity of calcineurin and an increased cytoplasmic concentration of phosphorylated NFAT; additionally, the

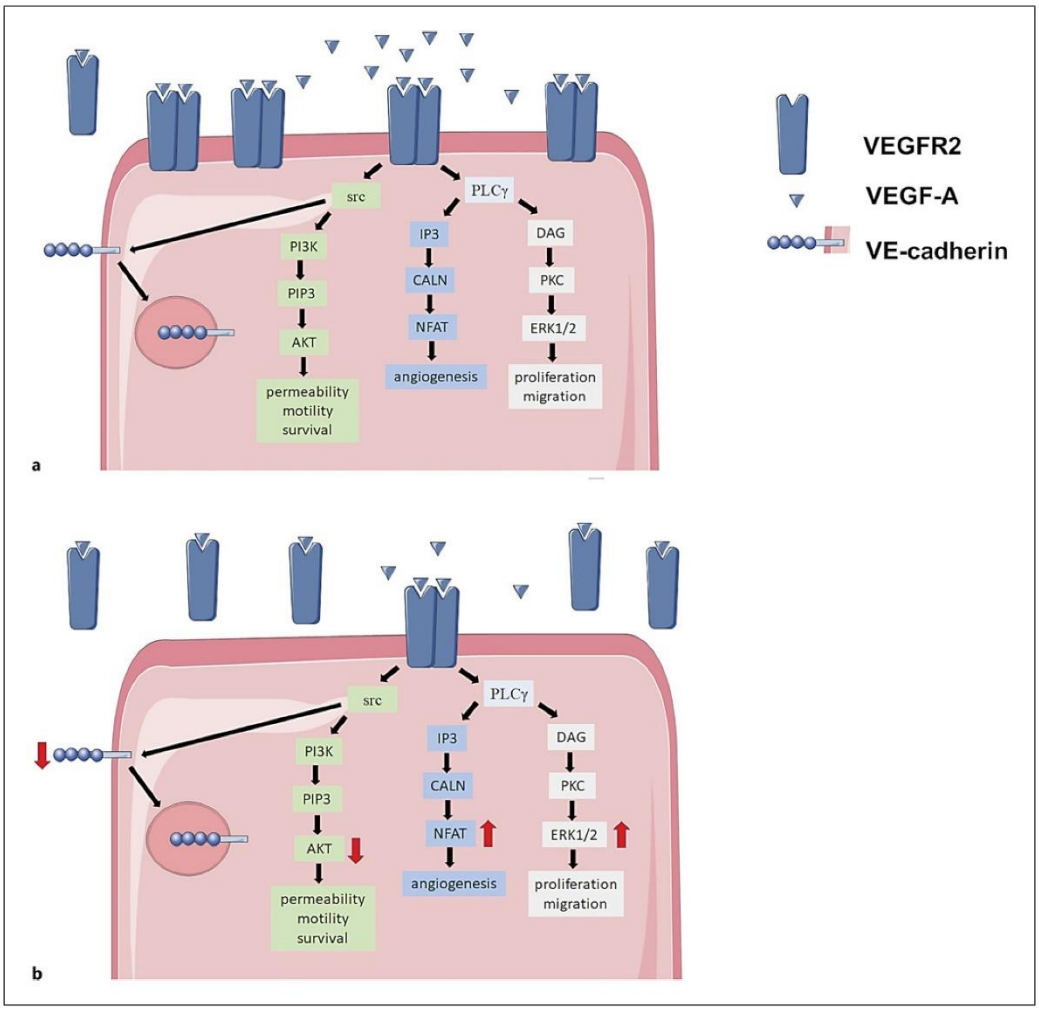

Fig. 1. VEGF-A-VEGFR2 signaling cascade in normal (**a**) and MetS/obesity (**b**) conditions. **a** Main signaling molecules involved in signal transmission via the VEGF-A-VEGFR2 and VE-cadherin axis. **b** MetS/obesity conditions affect the VEGF-A/VEGFR2/VE-cadherin signaling axis: (1) reduce AKT pathway activity and upregulate the PLC-γ pathway; (2) reduce the expression of VE-cadherin and elevate the internalization of these molecules.

amount of activated NFAT was reduced in the cell nucleus, which results in alteration of myofilament protein expression, disruption of sarcomere, and atrial myopathy [60]. Based on the scarce data regarding the VEGFR signaling in the heart in MetS, the ERK pathway is mostly upregulated, but further studies in this field are needed to assess the role of ERK activation, especially in the cardiac endothelium.

PI3K/AKT

In the hearts of db/db mice with MetS, a decrease in the concentration of active AKT was noted [52]. No difference in the AKT activity was observed, when only cardiomyocytes were analyzed, which suggests that this pathway may be affected in other cell types, such as fibroblasts or ECs [55]. Similarly, AKT phosphorylation is reduced in the hearts of ZDF rats, known to present MetS symptoms [57]. Diminished AKT phosphorylation was also confirmed in a diet-induced rat MetS model [61]. High-fructose diet results in the development of MetS symptoms and reduces AKT activity [62]. In another animal model (JCR:LA-cp rats), AKT activation failed in the heart during repetitive ischemia, which may explain diminished collateral formation; the observed phenomenon was linked to an altered REDOX state in MetS heart since when the levels of ROS were controlled, also the activity of AKT was restored [63]. Furthermore, in a high-cholesterol diet swine model, reduced cardiac phosphorylation of AKT was observed [64]. Some contradictory data are also available. In a porcine model of myocardial infarction, the cardiac AKT signaling pathway was activated in MetS, unlike that in the control group [59]. To sum up, the AKT signaling pathway in MetS is presumably downregulated or even blocked, which may exert a detrimental effect on the angiogenic response of ECs.

Src/VE-Cadherin

VE-cadherin phosphorylation, which is regulated via VEGF/VEGFR2/Src axis, disrupts EC adherens junctions and promotes vascular leakage [65]. On the other hand, destabilization of adherens junctions is an essential initial step of EC migration, necessary for new vessel formation [66]. Additionally, VE-cadherin may interact with VEGFR2 to prolong these receptors' half-life, and it has a positive impact on EC survival [67]. In a high-fat diet (HFD) porcine model, VE-cadherin expression is reduced after myocardial infarction, which indicates angiogenic pathway disruption and may not only promote vascular leakage but also diminish the proangiogenic response of ECs [68]. Of note, downregulation of VE-cadherin expression may be also caused by elevated numbers of neutrophils in cardiac tissue, as was described in a HFD mouse model [69]. Neutrophil infiltration in MetS may negatively affect collateral growth in MetS myocardium; one of the possible mechanisms may be an extensive release of neutrophil elastase that promotes VE-cadherin degradation, inhibits VE-cadherin expression, and causes vascular leakage [12, 69].

Src/p38 MAPK/AKT

Src, p38 MAPK, and AKT pathway activation, crucial for collateral growth in cardiac tissue, is redox dependent, and since ROS levels are elevated in MetS, an abnormal activation of above molecules may, at least in part, be responsible for compromised new vessel formation [63, 70, 71]. In ZDF rats and in db/db mice, phosphorylation of p38 MAPK was elevated in obese and diabetic animals. In this experiment, examination was performed on the whole cardiac tissue. Since the sample consisted mostly of cardiomyocytes and elevated p38 MAPK activity may correlate with inflammatory processes, these data probably do not describe events occurring in ECs [56, 57]. Activation of the p38 MAPK pathway in the aorta and coronary arteries was increased in MetS swine [64]. Summarizing, MAPK phosphorylation and thus activation is probably reduced in MetS hearts, although data are contradictory. Activation of p38 MAPK is strictly connected with REDOX state of the ECs, and an angiogenic response to VEGF is only possible when ROS levels are controlled. Altered activation of transcription factors regulated by phosphorylated MAPK proteins was also observed; for example, STAT-3 activation is reduced in the cardiac tissue of db/db mice [56]. Main VEGF-VEGFR signaling pathway alterations are summarized in Figure 1b.

Conclusion

There are a growing number of individuals with MetS worldwide. These patients develop HF, whose exact pathophysiology, especially in terms of vessel/capillary rarefaction associated with insufficient angiogenesis, is not fully understood. Moreover, it becomes increasingly problematic to diagnose and treat this complex disease that generates huge socioeconomic costs and markedly impairs health-related quality of life. MetS etiology and symptom progression leading to HF seem to be associated with adverse alterations in multiple metabolic pathways, leading to disrupted EC homeostasis (EC dysfunction) and impaired angiogenesis, followed by micro-vessel regression and collateral growth delay, all of which are recognized as early unfavorable pathological remodeling of cardiac muscle during MetS. Dysfunction of receptors for various VEGF molecules and their secondary messengers is partially responsible for impaired angiogenesis, reduced blood capillary density, and increased microvascular permeability in the hearts of

individuals suffering from MetS symptoms. Poor angiogenesis mediated by the VEGF/VEGFR axis is largely unknown in MetS and should be further investigated. Elucidation of the precise mechanisms regulating angiogenesis in MetS would lead to a better understanding of the mechanisms behind MetS-induced cardiac dysfunction and would be a stepping stone in the search for novel therapies of this clinical condition.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

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6.2. Praca: Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts.

Dzięki rzetelnemu przygotowaniu teoretycznemu możliwe było wykonanie doświadczeń, których wyniki zostały zaprezentowane w publikacji **Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts**. Wykorzystując mysi model zespołu metabolicznego opartego na mutacji w receptorze dla leptyny (myszy db/db) skupiono się na zaburzeniach jakie są obserwowane w naczyniach krwionośnych w sercu. Przeanalizowano obrazy morfologiczne przy zastosowaniu mikroskopii konfokalnej oraz ultrastrukturę w transmisyjnej mikroskopii elektronowej. Przeprowadzono analizę wybranych mRNA zaangażowanych w regulację angiogenezy oraz przepuszczalności naczyń krwionośnych w izolowanych komórkach śródbłonka. Przedstawiono zmiany ekspresji VE-kadheryny oraz VEGFR-2 w endothelium poprzez analizę obrazów mikroskopii konfokalnej. Ponadto przeprowadzono test stymulacji krążków aortalnych myszy kontrolnych i db/db celem oceny odpowiedzi angiogennej śródbłonka.

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ORIGINAL PAPER

Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts

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Abstract
Metabolic syndrome (MetS) is a condition that includes symptoms, such as obesity, hyperglycemia, and hypertension, which elevate cardiovascular risk. An impaired angiogenic response of endothelial cells (ECs) in heart and peripheral organs has been proposed in MetS, but the mechanisms of this phenomenon have not been thoroughly explored. Results obtained from evaluating the whole myocardium are inconsistent, since different types of cells react differently to MetS environment and a variety of molecular pathways are involved in the angiogenic response. Therefore, the aim of this paper was to study one selected pathway—the VEGF/VEGFR pathway, which regulates the angiogenic response and microvascular permeability in ECs isolated from db/db mouse hearts. The expression of mRNAs for VEGF/VEGFR axis proteins was assessed with RT-PCR in ECs isolated from control and db/db mouse myocardium. The density of CD31-, VEGFR2-, and VE-cadherin-positive cells was examined with confocal microscopy, and the ultrastructure of ECs was analyzed with transmission electron microscopy. The aortic ring assay was used to assess the capacity of ECs to respond to angiogenic stimuli. Our results showed a decreased number of microvessels, diminished expression of VE-cadherin and VEGFR2 and widened gaps between the ECs of microcapillaries. The aortic ring assay showed a diminished number of sprouts in db/db mice. These results may indicate that ECs in MetS enhance the production of mRNA for VEGF/VRGFR axis proteins, yet sprout formation and vascular barrier maintenance are limited. These novel data may provide a foundation for further studies on ECs dysfunction in MetS.

Keywords Angiogenesis · Heart failure · VEGF · VEGFR · Endothelial cell · Blood vessels

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Introduction

Obesity is a pandemic of modern times, since one-third of the human population is obese, if obesity is determined by a body mass index (BMI) of ≥ 30. Obesity is a major health care problem, as treatment of its complications is costly, and the number of obese people is increasing constantly (Meldrum et al. 2017). Obese people suffer from many diseases and exhibit a 60% higher risk of death (Afshin et al. 2017). Obesity, especially of the abdominal type, increases also the risk of cardiovascular disease and cardiovascular events by a factor of five (Ortega et al. 2016). Furthermore, obese people very often develop hypertension, hyperglycemia, and dyslipidemia. These symptoms and conditions are collectively referred to as metabolic syndrome (MetS) (Sherling et al. 2017).

MetS environment may lead to accelerated atherosclerosis, fatty liver disease, kidney insufficiency, and/or heart failure (Fahed et al. 2022). Typically, MetS leads to the development of heart failure with preserved ejection fraction (HFpEF) (Purwowiyoto and Prawara 2021). Various detrimental changes within the heart are observed in HFpEF. These changes include cardiomyocyte hypertrophy, reactive oxygen species (ROS) formation, coronary microvessel dysfunction associated with edema, which leads to fibrosis, and an increased ventricular stiffness (Mohammed et al. 2015). Endothelial cell (EC) metabolism dysregulation may impair the angiogenic response and trigger cell death (Salvatore et al. 2022). This results in insufficient nutrition supply and hypoxia, with the latter being a key factor in the development of cardiac inflammation and progression of heart failure (Saltiel and Olefsky 2017; Simmonds et al. 2020).

Impaired angiogenesis in MetS hearts has been well documented, but the causative mechanisms have not been precisely explored (Mazidi et al. 2017; Cheng et al. 2023; Bartkowiak et al. 2024). Formation of new blood vessels is regulated mainly via proangiogenic factors, such as VEGF or FGF, and by their receptors (VEGFR and FGFR), as well as by costimulatory molecules, such as neuropilins (Karaman et al. 2018; Peach et al. 2018). Receptor activation triggers intracellular downstream signaling pathways, among which the Akt and MAP pathways are crucial for events necessary for an angiogenic response, such as cell proliferation, cell migration, and sprout formation. Regulation of vascular permeability is also dependent on VEGF receptors but proceeds via a separate signaling pathway, namely via AKT and/or SRC activation (Simons et al. 2016). EC dysfunction in MetS has been linked to oxidative stress and metabolic deregulation of ECs, which in turn affect vascular tone, permeability, angiogenesis, and surface molecule expression, and may subsequently trigger leukocyte adhesion, inflammation, and EC death (Liu et al. 2023; Veitch et al. 2022).

Apart from VEGFs and their receptors, there are many other proangiogenic factors, notably leptin, which is able to exert effects similar to those of VEGF-A in vitro (Tahergorabi and Khazaei 2015). Leptin is an adipokine produced mostly within white adipose tissue, and it exerts both central and peripheral effects. This adipokine not only regulates energy homeostasis via central appetite regulation, but is also involved in reproduction, homeostasis, and immune function, via leptin receptors (LEPRs), which are found on various types of cells (Wauman and Tavernier 2011). MetS is associated with elevated serum leptin levels, which generates hypothalamic resistance to leptin signaling. There is also some data on peripheral leptin resistance in the heart owing to reduced LEPR expression or inactive LEPRs (Ren et al. 2008; Leifheit-Nestler et al. 2013). Leptin signaling malfunction is also observed in the ECs of obese individuals. It occurs either owing to LEPR downregulation or alterations in receptor binding sites within ECs. Abnormal leptin signaling may lead to an inflammatory response and atherosclerosis, but the mechanism in which the altered leptin signaling impacts cardiac microvessel angiogenesis has not been fully understood (Raman and Khanal 2021).

Our data show significant ultrastructural changes within the microvasculature of cardiac muscle obtained from db/db mice. These changes include increased gaps between ECs and a decreased vesicular index. Additionally, the number of vessels is decreased in db/db mouse myocardium. This may be a result of abnormalities in main signalling pathways involving VEGFR2 and its downstream molecules. Cardiac tissue consists of many different types of cells, among which cardiomyocytes predominate. ECs respond differently to an unfavorable MetS environment. Therefore, the aim of this study was to assess the expression of proangiogenic factors and their receptors in the whole myocardium of db/db mice and in isolated cardiac ECs; we also evaluated intracellular signaling pathways that regulate new blood vessel formation and vascular permeability. Db/db mice are considered to be an animal model of MetS (Fellmann et al. 2013; Alex et al. 2018).

Methods

Animals

BKS.Cg-Dock7^{m}+/+Lepr^{db}/J male mice (db/db; strain no. 000642, breeding location: Italy, order no. BKDSIMA09SS) and C57BL/6J (strain no. 000664, breeding location: Germany, order no. B6JSIMA09W) control mice were used for our experiments. All animal experiments had been approved by the First Local Committee for Ethics in Animal

Experiments in Warsaw (at the University of Warsaw, Poland, license no. 140/2016). Nine-week-old male mice were purchased from Charles River Laboratory (251 Ballardvale Str., Wilmington, MA 01887, USA) and kept under specific pathogen-free conditions, 12/12 h dark/light cycle, at 20–24 °C, with access to water and LabDiet® 5K52 (6% fat) chow (Charles River Laboratory, 251 Ballardvale Str., Wilmington, MA 01887, USA) ad libitum. The animals were sacrificed at the age of 21 weeks by CO_2 asphyxiation, and their hearts and aortas were isolated for further experiments.

Cardiac EC isolation by magnetic sorting

The hearts collected from 21-week-old db/db and control mice were cut in half, and rinsed in phosphate buffered saline (PBS). Next, the hearts were cut into pieces and digested with 0.5 mg/mL collagenase type II (Sigma-Aldrich, 3300 Str., St. Louis, MO 63118, USA) on a magnetic stirrer at 37 °C for 45 min. To obtain single cell suspensions, the digested tissue was pipetted and filtered through 40-µM nylon filters (Corning, 60 O'Connor Rd., Fairport, NY 14450, USA), and the cells were then washed with PBS and sorted with a magnetic-activated cell sorting system (MACS, Miltenyi Biotec, Inc., 2303 Lindbergh Str., Auburn, CA 95602, USA), according to the manufacturer's instructions. In brief, the cells were first incubated with primary rat antibodies against CD31 (cat. no 550274, BD Pharmingen Inc., 10975 Torreyana Rd., San Diego, CA 92121, USA) and CD45 (cat no. 550539, 10975 Torreyana Road San Diego, CA 92121 United States). Next, they were incubated with secondary anti-rat IgG antibodies conjugated with microbeads (cat no. 1300-048-501, Miltenyi Biotec, Inc., 2303 Lindbergh Str., Auburn, CA 95602, USA). After being labeled with the antibodies, the cells were separated with MACS Columns and Separators (Miltenyi Biotec, Inc., 2303 Lindbergh Str., Auburn, CA 95602, USA). First, the cells were subdivided into two populations: CD45-positive and CD45-negative. Subsequently, CD31-positive cells were isolated from the latter population. The obtained cells were immediately lysed with lysis buffer. Cells from one heart were collected for reverse transcription polymerase chain reaction (RT-PCR).

Total RNA isolation, reverse transcription (RT), and Real-Time PCR

In total, 30 mg tissue samples taken from mouse myocardia were transferred to lysis buffer and homogenized. Isolated cells were washed with ice-cold PBS and suspended in lysis buffer. Total RNA was isolated with a NucleoSpin ®RNA II kit (Macherey–Nagel, Valencienner Str. 11, 52355 Düren, Germany) according to the manufacturer's protocol. The concentration and purity of RNA were estimated with a NanoDrop spectrophotometer (NanoDrop Technologies LLC, 3411 Silverside Rd., Bancroft Building Wilmington, DE 19810, USA). Then, 500 ng of total RNA was reverse transcribed with a High-Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, 168 Third Ave., Waltham, MA 02451, USA), according to the manufacturer's protocol. cDNA was stored at −20 °C for further experiments. Gene expression was measured with relative quantitation (RQ) with a comparative CT assay. Real-Time PCR was performed with Abi Prism 7500 (Thermo Fisher Scientific, 168 Third Ave., Waltham, MA 02451, USA) in 96-well optical plates. Each sample was run in triplicate, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Mm99999915_g1) was used as an endogenous control. TaqMan gene expression assays (Thermo Fisher Scientific, MA, USA) were used to measure mRNA for selected genes: PLCγ Mm00549418_m1, cat. no. 4448892; PKCβ2 Mm00435749_m1, cat. no. 4448892; RAF1 Mm00466513_m1, cat. no. 4448892; MEK Mm00488759_m1, cat. no. 4448892; TSAd Mm00451631_m1, cat. no. 4448892; VEGF-A Mm00437306_m1, cat. no. 4453320, VE-cadherin Mm00486938_m1, cat. no. 4453320; eNOS Mm00435217_m1, cat. no. 4453320; VEGF-B Mm00442102_m1, cat. no. 4453320; VEGFR1 (Flt1) Mm00438980_m1, cat. no. 4453320, ERK Mm00442479_m1, cat. no. 4453320; Ki67, cat. no. Mm01278617_m1, cat. no. 4453320; FOXO1 Mm00490671_m1, cat. no. 4453320; FOXO3a Mm01185722_m1, cat. no. 4453320; PTEN Mm00477208_m1, cat. no. 4453320; PIK3ca Mm00435673_m1, cat. no. 4448892; SRC Mm00436785_m1 4448892; FAK Mm00433209_m1, cat. no. 4448892 PXN; Mm00448533_m1, cat. no. 4448892; VEGFR2 Mm01222421_m1, cat. no. 4453320; AKT1, Mm01331626_m1, cat. no. 4453320. All primer and probe sets were purchased from Thermo Fisher Scientific (168 Third Ave., Waltham, MA 02451, USA). The reactions were run with TaqMan Universal Master Mix (Thermo Fisher Scientific, 168 Third Ave., Waltham, MA 02451, USA), primer sets, a MGB probe, and a cDNA template (5 ng per reaction) in the universal thermal conditions 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C). The data were analyzed with sequence detection software version 1.4 (Thermo Fisher Scientific, 168 Third Ave., Waltham, MA 02451, USA).

Confocal microscopic evaluation of cardiac blood microvessels

Myocardial 10 µm-thick cryosections were fixed in buffered 4% paraformaldehyde solution, and immunostained with primary antibodies in various combinations, followed by incubation with secondary antibodies, according to published protocols (Flaht-Zabost et al. 2014). Primary

antibodies used were: anti-CD31 (cat no. 550274, BD Pharmingen Inc., 10975 Torreyana Rd., San Diego, CA 92121, USA), final dilution 1:100; anti-VE-cadherin (cat no. AF1002, R&D Systems, Inc., 614 McKinley Place NE, MN 55413, USA), final dilution 1:25; anti-VEGFR2 (cat no. ab51873, Abcam Limited, Discovery Drive, Cambridge Biomedical Campus, Cambridge, CB2 0AX, UK), final dilution 1:25, anti-alfa-SMA (cat no. ab21027, Abcam Limited, Discovery Drive, Cambridge Biomedical Campus, Cambridge, CB2 0AX, UK), final dilution 1:100; and anti-Lyve-1 (cat no. 11-034, Angiobio, Insight Biotechnology Limited PO Box 520. Wembley Middlesex HA9 7YN, UK), final dilution 1:300. Secondary antibodies were: donkey anti-rat immunoglobulin G (IgG) Alexa Fluor 647-conjugated, final dilution 1:500 (cat no. 712-605-153, Jackson ImmunoResearch, 872 W Baltimore Pike, West Grove, PA 19390, USA); donkey anti-goat IgG Fluorescein isothiocyanate (FITC)-conjugated (cat no. 705-095-147, Jackson ImmunoResearch, 872 W Baltimore Pike, West Grove, PA 19390, USA), final dilution 1:200; and donkey anti-rabbit IgG CyTM3-conugated, (cat no. 711-165-152, Jackson ImmunoResearch, 872 W Baltimore Pike, West Grove, PA 19390, USA), final dilution 1:800. Cell nuclei were counterstained with Hoechst (Sigma-Aldrich, 3300 Str., St. Louis, MO 63118, USA), according to the manufacturer's protocol. Specimens were mounted in fluorescence mounting medium (Dako, Produktionsvej 42, 2600 Glostrup, Denmark). Sections were viewed under confocal microscopes: Olympus FV 1000 (with oil immersion 20×/0.80 objective, images were analyzed with FV1000 Operations Software ver 4.2; Olympus, 2951 Ishikawa-machi, Hachioji-shi, Tokyo 192-8507, Japan), Leica TCS SP5 (with HCX PL APO L 20 × 1.0 water immersion objective, images were analyzed with LasAF Lite 3.3 software; Leica Microsystems GmbH. Ernst-Leitz-Strasse 17-37. 35578 Wetzlar Germany) or Zeiss LSM 780/Elyra PS.1 (with Plan-Apochromat 20×/0,8 objective, images were analyzed with ZEN 2.3; Carl Zeiss, Carl-Zeiss-Straße 22, 73447 Oberkochen, Germany). At least three independent immunostainings were performed on separate tissue sections. Myocardial sections were stained with each respective primary antibody and were used as positive controls. Separate sections without primary antibody treatment were used as negative controls. Photomicrographs show representative cross sections of the cardiac muscle. Microvessel density was evaluated only in regions where cardiomyocytes were transversely cut and at least six regions were randomly selected for calculations. Microvessel density has been presented as the number of $CD31^+$/Lyve-1^- vessels per 1 mm^2 of myocardial tissue sections. The density of VE-cadherin and VEGFR2 signal was calculated in relation to $CD31^+$/Lyve-1^- vessel density.

Ultrastructure and morphometric analysis of cardiac microvessels

Karnovsky-fixed (half-strength of Karnovsky fixative contains 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M of phosphate buffer, pH 7.4) small tissue specimens from mouse hearts were postfixed in 1% buffered osmium tetroxide and processed to Epon embedding (Polysciences, Inc. 400 Valley Road. Warrington, PA 18976, USA), according to a published protocol (Dobrzynska et al. 2015). Ultrathin sections were cut, contrasted with uranyl acetate and lead citrate, and examined by transmission electron microscopy (TEM) (Jeol JEM 1011; Jeol Ltd., 3-1-2 Musashino, Akishima, Tokyo 196-8558, Japan). Dimensions of gaps in areas of intercellular junctions between blood endothelial cells of cardiac microvessel and the number of cytoplasmic vesicles per 1 µm of cellular membrane length (specified here as vesicular indexes) were assessed and measured with a TEM morphometric program (iTEM Olympus, 2951 Ishikawa-machi, Hachioji-shi, Tokyo 192–8507, Japan).

Aortic ring assay

Collagen-coated culture chambers (Nunc™ Lab-Tek™ II Chamber Slide™ System, Thermo Fisher Scientific, 168 Third Ave., Waltham, MA 02451, USA) were prepared 2 h prior to aorta isolation; 450 µl of collagen solution (STEMCELL Technologies, 1618 Station Str. Vancouver, BC, V6A 1B6, Canada) was mixed on ice with 800 µl of ECM medium supplemented with 1% fetal calf serum (FCS), 1% antibiotic/antimycotic, EC growth supplement of 0.004 ml/ml, EGF of 0.1 ng/ml, bFGF of 1 ng/ml, heparin of 90 µg/ml, and hydrocortisone of 1 µg/ml (cat No. C-22110, PromoCell GmbH, Sickingenstr. 63/65, 69126 Heidelberg, Germany) and immediately transferred to the culture-chamber slide. After 1 h in a 37 °C incubator, the collagen was washed and soaked with ECM medium, supplemented as above. Aortas were isolated from the mice, cleared of adipose tissue, and cut into approximately 1 mm thick rings. Before the aorta rings were placed on the collagen, the culture medium was carefully removed to enable contact with the collagen surface. A medium enriched with mouse VEGF-A_{164} (50 ng/ml, from R&D Systems, Inc., 614 McKinley Place NE, MN 55413, USA) was added to the aorta culture after 18 h. The medium was changed every other day. Cultures were examined under a contrast-phase microscope every other day. After 8 days, the explants were fixed for immunostaining or dissected from the collagen, and remaining sprouts were lysed for RNA isolation.

Whole-mount immunostaining of aortic sprouts

Cultures of aortic rings were fixed with buffered 4% paraformaldehyde overnight at 4 °C, washed with PBS, and incubated with 1% bovine serum albumin (BSA), 0.1% TritonX-100, and 0.1 M of glycine in PBS for 30 min. After thorough rinsing with PBS, nonspecific background staining was blocked with 5% donkey serum (Jackson ImmunoResearch, 872 W Baltimore Pike, West Grove, PA 19390, USA). Subsequently, specimens were incubated overnight at 4 °C with a primary antibody: anti-CD31 (cat no. 550274, BD Pharmingen Inc., 10975 Torreyana Rd., San Diego, CA 92121, USA) diluted in PBS/1% BSA 1:100. After three washes in PBS, specimens were incubated for 3 h with donkey secondary antibodies diluted in PBS/1% BSA conjugated with a fluorescent particle (donkey anti-rat IgG AlexaFluor® 647, dilution 1:500, cat No. 712-605-153, Jackson ImmunoResearch, 872 W Baltimore Pike, West Grove, PA 19390, USA), washed as above, and cell nuclei were counterstained with Hoechst (Sigma-Aldrich, 3300 Str., St. Louis, MO 63118, USA), according to the manufacturer's protocol. Specimens were mounted in fluorescence mounting medium (Dako, Produktionsvej 42, 2600 Glostrup, Denmark) and viewed under a Leica confocal microscope, type TCS SP5, with HCX PL APO L 20 × 1.0 water immersion objective. Images were analyzed with LasAF Lite 3.3 software (Leica Microsystems GmbH. Ernst-Leitz-Strasse 17-37, 35578 Wetzlar, Germany). During analysis, the number of sprouts per explant was calculated.

Statistical analysis

Data were analyzed with GraphPad Prism 9 (GraphPad Software, 2365 Northside Dr., Ste. 560, San Diego, CA 92108, USA). The normality of distribution was assessed by the Shapiro–Wilk test, then the *t*-test or the Mann–Whitney test was applied. Results were considered statistically significant at a p-value of ≤ 0.05.

Results

In db/db mice the density of microvessels is reducted, and the integrity of the microvascular barrier is compromised

Microvascular density, calculated as a number of $CD31^+$/Lyve-1^- cells per one square mm of tissue, was evaluated in the hearts of db/db mice and those of controls (Fig. 1). Since experimental animals showed no more cardiomyocyte hypertrophy than controls (data not shown), the number of vessels was counted per unit of area. In the experimental group statistically significant decrease in $CD31^+$/Lyve-1^- cells in the whole heart, as well as in separate areas of the heart, e.g., the interventricular septum, left ventricle, and right ventricle, were observed (Fig. 1c–f).

Widened junctional gaps between ECs are considered a morphological sign of increased vascular permeability (McDonald et al. 1999). Figure 2a–d illustrates EC morphology in cardiac microcapillaries. The distances between adjacent ECs, i.e., junctional gaps and cytoplasmic vesicles, in blood capillary ECs are shown on Fig. 2a–d. Analysis of gaps with TEM displayed an increased width in db/db in comparison with that in control mice (Fig. 2e–g) at the base and at the apex of the heart. No difference was observed within the middle region of the heart. Furthermore, we observed a decreased vesicular index at the apex of the heart, but not in other regions. This may indicate that active transcellular transport was decreased in the region of the heart apex (Fig. 2h–j).

The expression of mRNA for crucial receptors and growth factors involved in regulation of angiogenesis and vessel integrity is upregulated only in isolated ECs from db/db mice, but not in the whole myocardium

To further analyze the possible mechanism leading to vascular rarefaction and leakiness in db/db mice, the expression of VEGF-A, VEGF-B and VEGFR1, and VEGFR2 in whole cardiac muscle and in isolated ECs were evaluated. There were no statistical differences in the expression of mRNA for VEGF-A, VEGF-B, VEGFR1, or VEGFR2 in the whole cardiac tissue of control and db/db groups (Fig. 3a–d). However, in isolated ECs there was a significantly increased expression of VEGF-A, VEGF-B, and their receptors in the db/db group (Fig. 3e–h). In situ evaluation of VEGFR2 expression showed a significant downregulation of this protein in whole heart, which may reflect the observed decrease in the number of microvessels (Fig. 4a–f). Additionally, VEGFR2/CD31 ratio analysis showed a decreased expression of VEGFR in whole heart and in the interventricular septum, but not in the left or right ventricle (Fig. 4g–j).

The expression of mRNA for the MAP-signalling pathway is elevated in isolated cardiac ECs from db/db mice

To evaluate the possible irregularities in the downstream VEGFR2 signalling cascade, we examined the expression of mRNA for main molecules involved in the MAP-regulated pathway. Only the expression of mRNA for MEK and ERK was significantly increased in the whole heart in db/db mice. Despite this, the expression of mRNA for Ki67 was decreased in db/db hearts in comparison to those in the control group (Fig. 5a–f). In isolated ECs there was

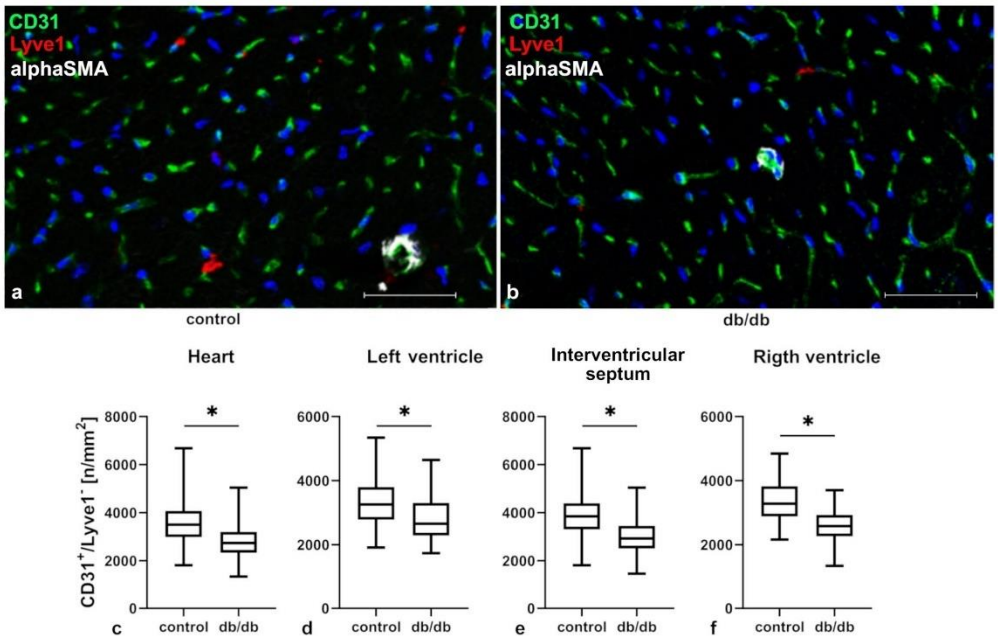

Fig. 1 Density of microvessels in cardiac tissue calculated as a number of $CD31^+/Lyve\text{-}1^-$ cells per one square mm of tissue in control and db/db mice. Panels **a**, **b** show confocal analysis of $CD31^+/Lyve\text{-}1^-$ cells; panels **c–f** show density of $CD31^+/Lyve\text{-}1^-$ cells per one square mm of tissue in various parts of the heart. Graphs **c–f** show measurements from three mouse hearts per group; at least six randomly selected regions of interest were analyzed for each location ($n = 18$); only transverse sections of tissue were included into calculations. The normality of the distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test were applied depending on data distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05. $^*p < 0.05$ Error bars show standard deviation of the mean

upregulation of mRNA for VEGFR2, PKCγβ2, PLCγ, RAF-1, MEK, and ERK (Fig. 5g–l), but the expression of Ki-67 was not affected.

Sprouting is impaired in the aortic ring assay with db/db-derived aortas stimulated with angiogenic factors

The aortic ring assay was performed with aortas isolated from db/db and control mice. Incubation with proangiogenic $VEGF\text{-}A_{164}$ isoform stimulated the formation of sprouts, defined as CD31-positive structures, but the number of sprouts was significantly lower in db/db-derived aortic rings compared with the number of sprouts from control-derived aortic rings (Fig. 5m–o). Furthermore, the mRNA levels for VEGFR2 showed no significant differences between the control and db/db groups, whereas mRNA for ERK1/2 molecules was significantly upregulated in db/db aortas supplemented with proangiogenic factors (Fig. 5p, q).

The upregulated expression of mRNAs for AKT1 signalling pathways are observed in ECs isolated from cardiac muscle of db/db mice

The expression of mRNA for proteins involved in the AKT signalling pathway in whole cardiac tissue was not significantly affected, except for FOXO3a, which was upregulated in db/db cardiac muscle when compared with control cardiac muscle (Fig. 6a–j). On the other hand, all examined mRNAs (SRC, FAK, PXN, VE-cadherin, AKT1, PI3K, eNOS, FOXO1, FOXO3a, and PTEN) were significantly upregulated in isolated cardiac ECs from db/db mice (Fig. 6k–t). The in situ expression of VE-cadherin, evaluated with confocal microscopy, was downregulated in the whole heart and in both ventricles, but not in the interventricular septum, which may be due to a decrease in the number of microvessels in the ventricles of db/db mice (Fig. 7a–f). The VE-cadherin/CD31 ratio showed a decrease of VE-cadherin expression in ECs only in the left ventricle. There was no change in

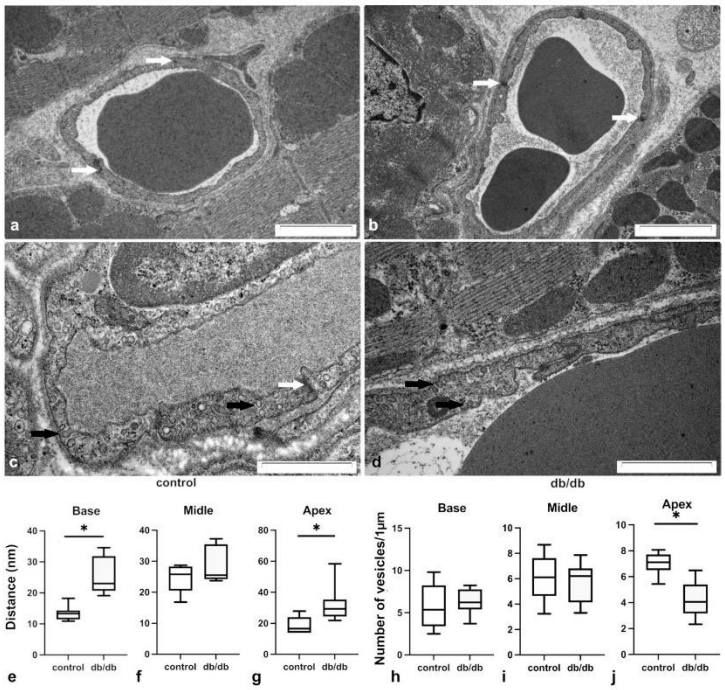

Fig. 2 Cardiac blood microcapillary remodeling. TEM details of microcapillaries in a control (**a**, **c**) and db/db (**b**, **d**) mouse. Distances between adjacent ECs (i.e., junctional gaps) and cytoplasmic vesicles in blood capillary ECs are shown in **a–d**, respectively. Junctional gap distances (**e–g**) and the number of vesicles (**h–j**) were calculated in different areas of the heart. Calculations were made on the basis of samples taken from three hearts per group. The number of randomly selected areas for measurements in each group were: (**e–g**) control—base $n=8$, middle $n=6$, apex $n=6$, db/db $n=45$, db/db—base $n=9$, middle $n=6$, apex $n=10$; (**h–j**) control—base $n=10$, middle $n=10$, apex $n=10$, db/db n = 45, db/db—base $n=10$, middle $n=10$, apex $n=10$, db/db. The normality of distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on the distribution. The results were considered statistically significant at a *p*-value of ≤ 0.05; *n*—number of regions of interest (randomly selected areas for measurements); white arrows—junctional gaps; black arrows—vesicles in EC cytoplasm. $^*p<0.05$. Error bars show standard deviation of the mean

VE-cadherin expression in the whole heart or in the interventricular septum, and an increase in VE-cadherin expression in ECs in the right ventricle (Fig. 7g–j).

Discussion

Number and morphology of microvessels in cardiac muscle of db/db mice

MetS symptoms, such as obesity, hyperglycemia, hyperlipidemia, and hypertension, negatively affect the cardiovascular system (Purwowiyoto and Prawara 2021). MetS conditions have been repeatedly reported to alter the expression of molecules involved in angiogenesis and vascular homeostasis, which in turn may contribute to some symptoms of this condition, such as delayed wound healing and impaired collateral growth after cardiac ischemia (Chou et al. 2002; Bartkowiak et al. 2024; Cheng et al. 2023). Moreover, unfavorable changes in cardiac vessel EC metabolism occur before the development of diastolic dysfunction in diabetic individuals (Taqueti et al. 2018). Similarly, microvascular rarefaction and dysfunction in peripheral organs are observed in animal models in prediabetic state, although the data are often contradictory (Veitch et al. 2022; Roy et al. 2022). There are two possibilities discussed—poor angiogenesis and increased apoptosis (Rawal et al. 2017; Niderla-Bielinska et al. 2021; Dham et al. 2021).

Db/db mouse is one of the animal models used for MetS studies (Alex et al. 2018). As a result of a point mutation, leptin receptors in db/db mice become inactivated; therefore, these mice develop central leptin resistance, causing

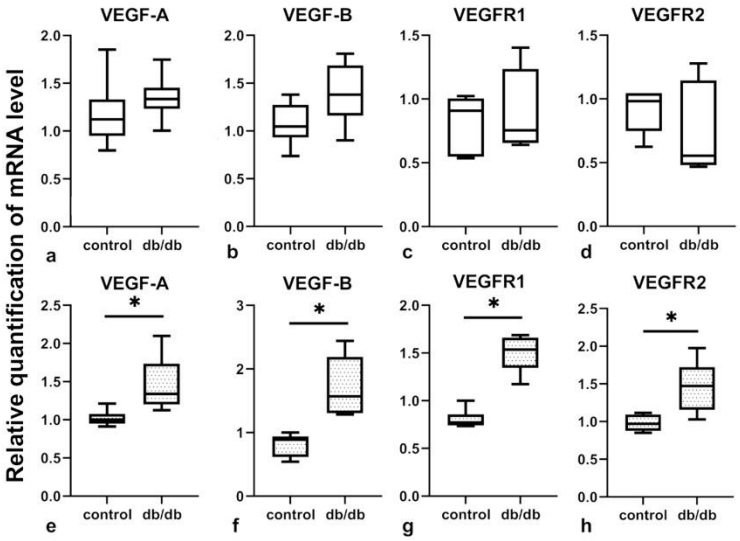

Fig. 3 Expression of mRNA for crucial growth factors and receptors involved in the regulation of angiogenesis in control and db/db mouse cardiac tissue and in isolated ECs ($n = 5$ for each group). Panels **a–h** show relative quantification of mRNA for selected molecules involved in angiogenesis regulation in whole cardiac tissue and in isolated ECs, respectively. The normality of the distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on data distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05; *n*—the number of mice used for whole cardiac tissue mRNA extraction or EC isolation and mRNA extraction. $^*p < 0.05$. Error bars show standard deviation of the mean

a voracious appetite and subsequent obesity and diabetes. Dysregulation of the central leptin axis is considered a main cause of obesity (Ren 2004), but in humans it is primarily overeating that rapidly increases blood leptin levels, leading to leptin resistance and weight gain (Ren 2004; Berger and Kloting 2021; Schwartz et al. 1996). Therefore, the results obtained in this study cannot be directly translated to patients, although there are some rare congenital LEPR or leptin mutations in humans that can lead to overeating, obesity, and T2DM (Farooqi et al. 2007). It is worth mentioning that there are also mouse models with EC-specific LEPR deficiency, in which the animals develop obesity only when high fat diet is introduced (Gogiraju et al. 2023).

Weight gain and hyperglycemia in db/db mice is associated with alterations in myocardial structure and function, although study results are often contradictory and depend heavily on mouse sex, age, and the specific db/db strain (Alex et al. 2018). In our experiment db/db mice at the of age 21 weeks were already developing severe obesity and hyperglycemia, although heart weight (calculated as heart weight/tibia length) was significantly lower when compared with that in the control group (data shown in (Niderla-Bielinska et al. 2021)). Morphological assessments of cardiac muscle revealed microvascular rarefaction and compromised vascular barrier. Although our first observation was consistent with previously published data (Alex et al. 2018; Rawal et al. 2017; Veitch et al. 2022), vascular barrier integrity in cardiac muscle was never before evaluated in db/db mice. Since we were able to employ only one method of assessing vascular permeability, which was morphological analysis of junctional gaps (McDonald et al. 1999), this observation needs further studies.

mRNAs for proteins involved in the angiogenic response are altered in ECs isolated from db/db mouse cardiac muscle

VEGFs are crucial proteins involved in regulation of angiogenesis. Elevated levels of VEGF-A in serum of MetS individuals are well documented and positively correlate with the amount of white adipose tissue (Zafar et al. 2018). In contrast, measurements of VEGF and VEGF receptor expression in cardiac tissue show contradictory results. No differences in the expression of VEGF-A, either on mRNA or protein levels were observed in the myocardium of db/db mice compared with controls (Broderick et al. 2012, 2019; Chen et al. 2012). Diabetic rats and diabetic patients show a decrease in VEGF-A and VEGFR2 expression in cardiac tissue (Chou et al. 2002). This may be owing to the fact, that these analyses involved whole cardiac tissue, which is composed of different types of cells that can respond to diabetic environment in different ways (Litviňuková et al. 2020). In our experiment RT-PCR analysis of mRNA expression for VEGF-A and VEGF-B in whole cardiac tissue did not show any changes between db/db and control animals. However, when we performed the same analysis with isolated ECs, we observed a statistically significant increase in mRNA for these factors. Furthermore, the expression of two crucial receptors for VEGFs (VEGFR1 and VEGFR2) also yielded surprising results. In the cardiac tissue there was

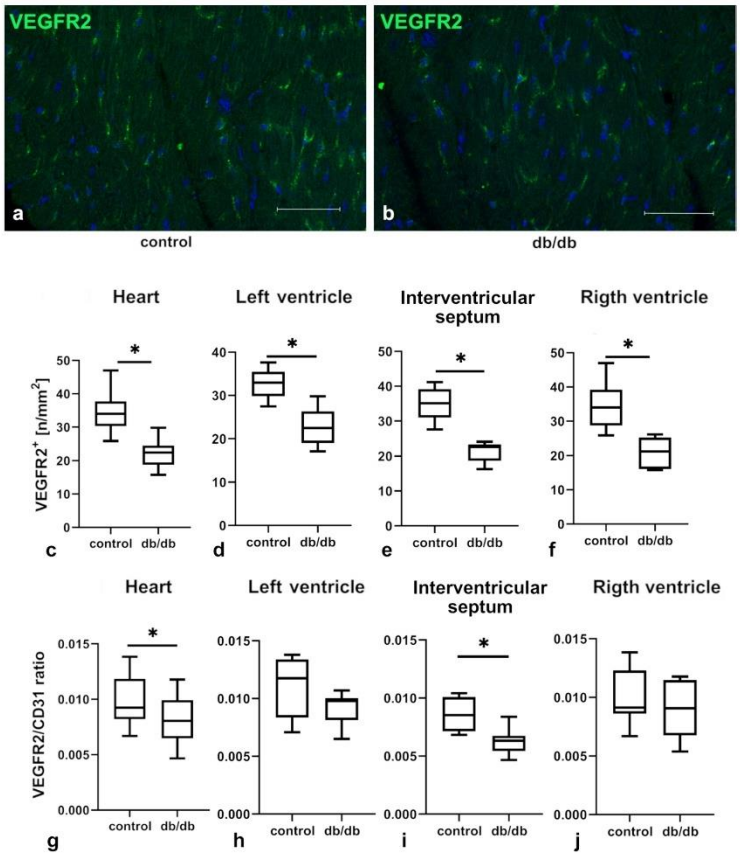

Fig. 4 The density of VEGFR2-positive cells in the whole heart and in its regions evaluated via confocal microscopy and measured per 1 mm^2 of tissue. Panels **a**, **b** show VEGFR2 immunostaining in control and db/db mice, respectively; graphs **c–f** show the number of VEGFR2-positive cells per 1 mm^2 of tissue in various regions of the heart; graphs **g–j** show the VEGFR2/CD31 ratio in various regions of the heart. **c-f** Show measurements from three mouse hearts per group; at least six randomly selected regions of interest were analyzed for each location ($n=18$); only transverse sections of tissue were included into calculations. The normality of distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on data distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05. $^*p < 0.05$. Error bars show standard deviation of the mean

no change in mRNA expression for these receptors, but when only the ECs are analyzed, increased expression was observed. This is in contrast to previously obtained results, where a reduction of VEGFR1 and VEGFR2 mRNA expression in the whole cardiac tissue in db/db mice was reported (Park et al. 2009). This may be owing to different age of the animals used in these experiments. In literature there is a lack of results showing mRNA expression in isolated ECs (Krüger-Genge et al. 2019). Unfortunately, we were unable to analyze the expression of VEGFs and their receptors on the protein levels owing to the small amount of the ECs obtained after isolation, but confocal microscopic analysis showed that the VEGFR2/CD31 positive signal ratio is decreased in the whole heart and in the interventricular septum. This may indirectly indicate a downregulation of this protein expression in ECs, despite elevation of the mRNA for VEGFR2. This may be due to excessive VEGFR2 trafficking and degradation with autophagy, which were previously observed in diabetic environment (Liu et al. 2012). Upregulation of VEGFR2 mRNA could be a compensatory mechanism to replace lost VEGFR2 proteins.

VEGFR2 function in ECs depends on the downstream signaling cascade. EC proliferation and differentiation, which are crucial for an angiogenic response, are mostly regulated via the PLCγ or ERK1/2 pathway (Claesson-Welsh 2016). In our experiments we observed an increased expression of mRNA for the proteins that are involved in this signaling pathway in whole heart tissue; however, the increase was not statistically significant, except for MEK and ERK1/2. On the other hand, in isolated ECs we observed a statistically significant elevation of mRNA expression for PLCγ, MEK, ERK, RAF1, and PKCβ2. In literature there are limited data describing the PLC-γ or ERK1/2 pathway in MetS hearts and the results are often contradictory. The

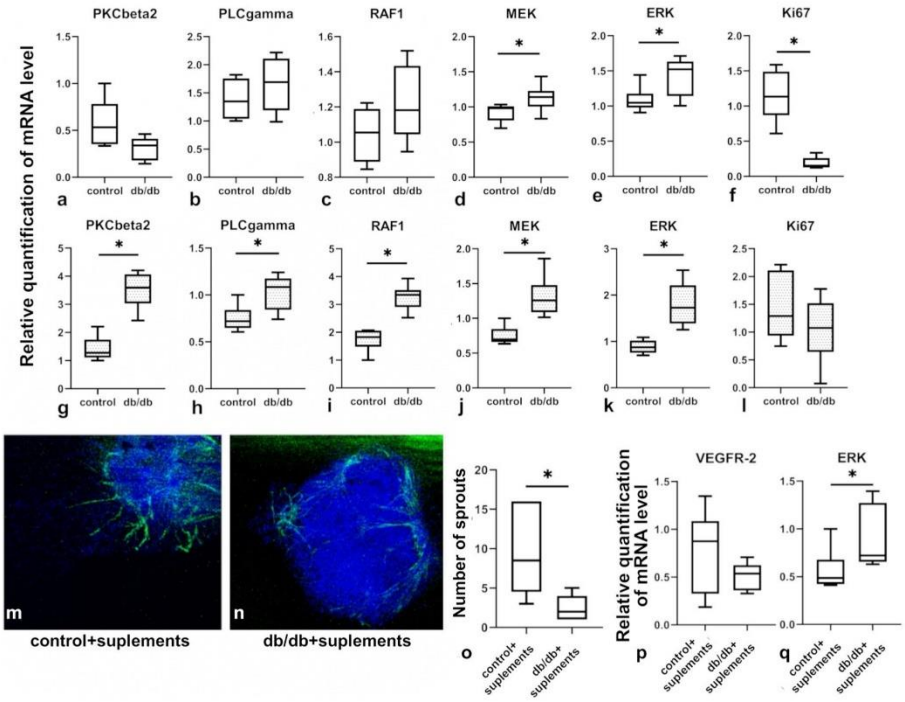

Fig. 5 The expression of mRNA for key molecules involved in the MAP-kinase pathway and the ability of ECs to respond to angiogenic stimuli. Panels **a–l** show relative quantification of mRNA for selected molecules involved in the MAP-kinase pathway in the whole cardiac tissue and in isolated ECs, respectively ($n=5$ for each group). Panels **m**, **n** show a representative result of the aortic ring assay; tubules were stained with anti-CD31 antibodies. Graph (**o**) shows the number of sprouts produced in control- and db/db-mouse-derived aortas under the influence of mouse VEGF-A_{165}. Panels **p**, **q** demonstrate relative quantification of mRNA for VGFR2 and ERK in vascular sprouts from the aortic ring assay. Panels **o–q** show measurements from three mouse experiments per group; at least five aortic rings per mouse were analyzed ($n=15$). The normality of distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on data distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05. $^*p<0.05$. Error bars show standard deviation of the mean

ERK1/2 pathway may be triggered in different types of cells, and results obtained from the whole cardiac tissue may be misleading with regard to the angiogenic response. Data show that in cardiomyocytes of db/db mice with MetS there are no major changes in the expression of ERK1/2 pathway kinases (Marsh et al. 2011). Similarly MetS conditions in a porcine model do not affect ERK1/2 expression or activation (Huang et al. 2013). On the other hand, in the whole myocardium increased activation of the ERK1/2 pathway was observed in db/db mice and in Zucker Diabetic Fatty rats, which also develop MetS conditions (Chen et al. 2018; Guleria et al. 2013). Elevated levels of phosphorylated ERK1/2 may correlate with elevated oxidative stress in aortic samples from obese rats (Touati et al. 2015). Taken together, our results suggest an upregulation of the mRNA expression for ERK signaling pathway molecules in the hearts in MetS, both in cardiac tissue and in isolated ECs. However, the latter may be an effect of elevated oxidative stress in these cells (Touati et al. 2015). Another explanation is that ERK1/2 pathway upregulation is a compensatory mechanism in ECs, which try to activate angiogenic pathways, but the EC dysfunction caused by MetS environment prevents it. Nevertheless, further experiments need to be performed to support these assumptions, since our evaluations were performed only with mRNA levels and these may not correspond with protein levels or the activation of specific molecules.

AKT-signaling pathway activation results in an increased expression of the mTOR2 complex, which is an important intracellular factor that facilitates new vessel formation by regulating cell proliferation, adhesion, and motility

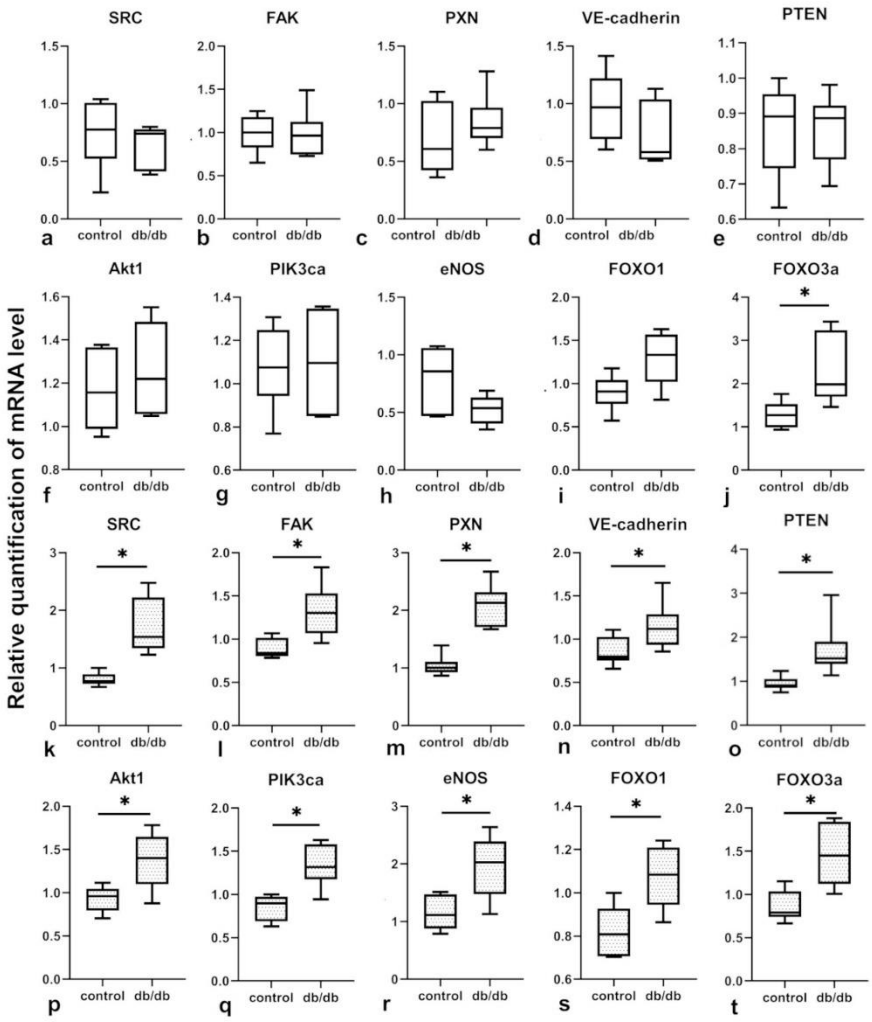

Fig. 6 mRNA expression for proteins involved in the AKT signaling pathway in the whole cardiac tissue (**a–j**) and in isolated ECs (**k–t**). Isolated cells and cardiac tissue samples were collected separately from five animals ($n=5$) in both the control and the db/db group. The normality of distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on data distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05. $^{*}p < 0.05$. Error bars show standard deviation of the mean

(Simons et al. 2016). There are many contradictory studies that describe AKT signaling in MetS (Park et al. 2009; Guleria et al. 2013). In this paper, we show, that there are no changes in mRNA expression for most AKT signaling cascade molecules (SRC, FAK, PXN, VE-cadherin, AKT1, PIK3ca, eNOS, FOXO1, and PTEN), except for FOXO3a, in the whole heart tissue, although for our experiments we did not choose any specific area of the heart, which may have affected our findings. On the other hand, all of the above factors are significantly elevated in isolated ECs. In the cardiac muscle of db/db mice the AKT pathway seems to be downregulated, as demonstrated by an observed decrease in phosphorylated AKT in tissue extracts (Park et al. 2009). Moreover, this effect is not observed in cardiomyocytes, which

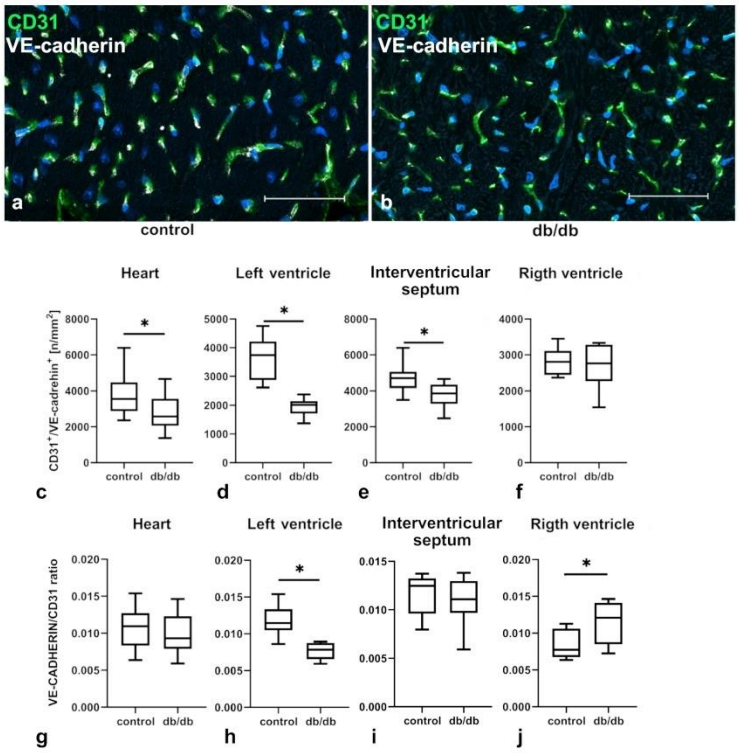

Fig. 7 VE-cadherin expression in control and db/db mouse hearts, analyzed with confocal microscopy (**a**, **b**). Graphs **c–f** show density of VE-cadherin and CD31 double-positive cells in the whole heart and in cardiac regions evaluated with confocal microscopy and measured per 1 mm^2 of tissue. Graphs **g–j** demonstrate the VE-cadherin/CD31 ratio in various regions of the heart. Graphs **c–j** show measurements from three mouse hearts per group; at least six randomly selected regions of interest were analyzed for each location ($n = 18$); only transverse sections of tissue were included into calculations. The normality of distribution was assessed by the Shapiro–Wilk test. The *t*-test or the Mann–Whitney test was used depending on distribution. Results were considered statistically significant at a *p*-value of ≤ 0.05. $^*p < 0.05$. Error bars show standard deviation of the mean

suggests that the AKT pathway may also be affected in other cells, such as fibroblasts, immune cells, or ECs (Marsh et al. 2011). Decreased AKT phosphorylation was also observed in a diet-induced MetS rat model (Ibarra-Lara et al. 2016).

In our experiments we also observed upregulated expression of mRNA for FOXO3a, a factor that enhances apoptosis in mature ECs via downregulation of antiapoptotic inhibitory proteins and attenuates EC proliferation (Skurk et al. 2004; Potente et al. 2003), both in whole cardiac tissue and in isolated ECs. FOXO3a negatively affects angiogenesis and EC proliferation by downregulating eNOS expression (Potente et al. 2005). We did not observe downregulation of mRNA for eNOS in isolated ECs, but the expression of mRNA for the Ki67 proliferation marker (Lashen et al. 2023) was decreased in the whole cardiac tissue. This may be at least partially owing to FOXO3a mRNA upregulation, although we did not observe the difference in mRNA expression for Ki67 in isolated db/db ECs, despite increased mRNA expression for FOXO3a.

In the next step we performed an aortic ring assay, which suggests, that new sprout formation is impaired in db/db mice, which was also reported earlier (Liu et al. 2012; Li et al. 2016). We also analyzed the mRNA for VEGFR2 and EKR1/2 molecules in aortic explants, which are crucial in regulating EC proliferation and angiogenesis, and we found that only ERK1/2 mRNA was upregulated in db/db mouse aortas treated with the proangiogenic factor ($VEGF_{164}$). Previous reports show that diabetic Akita mice exhibited a downregulation in VEGFR2 levels in an aortic ring model, but this may be owing to the fact that the VEGFR2 protein is degraded in cytosol, instead of being trafficked from endosomes to the plasma membrane, thus its mRNA levels may be unaffected (Liu et al. 2012).

Junctional gaps are widened in microvessels in db/db cardiac muscle

Vascular permeability is controlled by three mechanisms: interactions between ECs and mural cells, transcellular transport via vesicles, and paracellular transport between ECs, which depends on the integrity of cellular junctions (Goddard and Iruela-Arispe 2013; Miyawaki-Shimizu et al. 2006). Our ultrastructural analysis of db/db mouse microvessels within some regions of the heart showed widened

junctional gaps between ECs and a decreased vesicular index, which may suggest changes in microvessel permeability and/or an impaired microvessel barrier (Weis et al. 2004b; McDonald et al. 1999). VEGF downstream signaling pathway molecules, namely src kinases, as well as high levels of ROS, may affect transcellular trafficking through interaction with caveolin 1, the main protein involved in vesicle formation in ECs (Sun et al. 2009). Activation of the SRC signaling pathway results in VE-cadherin phosphorylation and endocytosis, destruction of adherents junctions, and increased endothelial permeability (Simons et al. 2016; Weis et al. 2004a). VE-cadherin may also couple with VEGFR2, and an increased phosphorylation of VE-cadherin causes uncoupling and weakening of cell-to-cell interactions and thus impairs the integrity of the vascular barrier (Shiou et al. 2019). Elevated levels of VEGF-A may additionally promote VE-cadherin phosphorylation via the VEGF-A/VEGFR/SRC axis (Weis et al. 2004b). In our work, we observed, that the expression of mRNA for VE-cadherin and VEGF-A is increased in ECs isolated from db/db mice but not in the whole heart. The VE-cadherin/CD31 ratio, analyzed with confocal microscopy, showed that VE-cadherin expression is downregulated in the left ventricle, but elevated in the right ventricle. This may indicate that different parts of the heart are affected by MetS environment in different ways. Additionally, elevated mRNA for VE-cadherin is insufficient to supplement the loss of VE-cadherin proteins.

LEPR deficiency in db/db mouse may be a possible cause of poor angiogenic response in cardiac muscle

VEGF–VEGFR signaling is not the only angiogenic pathway in the heart, although our experiments focused only on molecules involved in this axis. It needs to be mentioned that a poor angiogenic response may be also a result of the animal model used in our experiments, since db/db mice do not have functional receptors for leptin. Leptin is known to exert vascular effects, although its cardiovascular function remains controversial, since different, often contradictory, effects of this adipokine have been reported. Leptin may be involved in the regulation of vascular tone, arterial blood pressure, and vascular relaxation via different central and peripheral mechanisms (extensively described in (Ren 2004; Mellott and Faulkner 2023)]); therefore, defective leptin signaling may lead to pathological changes within myocardium, which can ultimately contribute to HF. Leptin may enhance angiogenesis, ECs survival and migration, and tube formation in vitro (Tahergorabi and Khazaei 2015) and in vivo, i.e., in a corneal angiogenic model or in a chorioallantoic membrane model (CAM) (Bouloumie et al. 1998; Park et al. 2001; Sierra-Honigmann et al. 1998). Leptin signaling promotes tube formation through interaction with the VEGFR2 downstream signaling cascade in cultured human umbilical vein ECs (HUVECs) (Garonna et al. 2011), whereas in cancer cells, LEPR activation stimulates VEGF-A expression mainly through hypoxia-inducible factor-1 α (HIF-1α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Gonzalez-Perez et al. 2010). LEPRs are present on ECs (Schroeter et al. 2007), but prolonged hyperleptinemia may alter LEPR expression, its distribution, or its downstream signaling cascade. Hubert et al. showed that mice fed a high-fat diet exhibit reduced LEPR expression and leptin-induced STAT3 phosphorylation (Hubert et al. 2017). Additionally, obesity may increase the levels of soluble LEPR, which limits leptin bioavailability (Hubert et al. 2017). Systemic LEPR deficiency affects all LEPR-expressing cells thus any cell-specific effects may be distorted. There are mouse models with reduced LEPR expression in ECs only, which mimic peripheral leptin resistance and show a vascular phenotype similar to that of HFD-fed WT mice (Hubert et al. 2017). On the other hand, deletion of LEPR in cardiac ECs promotes angiogenic sprouting in vitro, restores density of microvessels in cardiac muscle and reduces EC apoptosis after pressure overload (Gogiraju et al. 2019). Since db/db mice lack functional LEPR, we cannot rule out the possibility, that a poor angiogenic response in our aortic ring model was also a result of insufficient LEPR–VEGFR interactions, but this phenomenon needs further studies.

Final conclusions and limitations of the study

Expression of mRNAs for selected proangiogenic factors and the signaling molecules involved in angiogenesis, cell survival, and vascular permeability is altered in db/db mouse ECs isolated from cardiac muscle, whereas it remains unaltered when the entire cardiac tissue is analyzed. Our studies show that isolation and analysis of ECs is crucial for evaluating cellular processes, especially in cardiac muscle, where the presence of other cell types may obscure the view. Morphological changes within the cardiac muscle, namely microvascular rarefaction and widened vascular junctional gaps, may be caused by deregulated expression of mRNA for VEGFR signaling cascade molecules, but these are preliminary observations that need further studies. Our paper has some limitations, examination of such complicated processes as EC physiology and function in tissue environment is subject to error. We were unable to show the changes in selected molecules on the protein level or evaluate the activity of proteins involved in the VEGF signaling cascade owing to the limited number of cells sorted. But we believe that our findings are important for the following reasons: first, they demonstrate new aspects of VEGF signaling in ECs only, not in the whole cardiac tissue, in the MetS

environment, which are important from the point of view of clinical medicine; second, our findings may serve as a stepping stone for further research, including functional studies.

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Data availability No datasets were generated or analyzed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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7. Podsumowanie i wnioski

Przeprowadzone doświadczenia pozwoliły uzyskać następujące wyniki:

1. U myszy db/db gęstość mikronaczyń w całym miokardium jest istotnie obniżona
2. W badaniu ultrastrukturalnym zaobserwowano powiększenie odstępu pomiędzy komórkami śródbłonka, a także zmniejszoną intensywność transportu przez komórkę (transcytozy), co jest oznaką rozszczelnienia bariery naczyniowej. Obserwacje były istotne statystycznie jedynie dla niektórych obszarów serca
3. Ekspresja mRNA dla VEGF-A, VEGF-B oraz receptorów VEGFR-1, VEGFR-2 była istotnie zwiększona w izolowanych z serc myszy db/db komórkach śródbłonka. Efektu tego nie obserwowano, kiedy analizowano całe miokardium
4. Analiza poziomu ekspresji VEGFR-2 na skrawkach miokardium myszy db/db wykazała istotny statystycznie spadek gęstości tego receptora na komórkach śródbłonka, ale nie we wszystkich obszarach serca
5. Ekspresja mRNA dla PKCγβ2, PLCγ, RAF1, MEK, i ERK1/2 była zwiększona w izolowanych komórkach śródbłonka, natomiast ekspresja mRNA dla Ki-67, markera proliferacji, nie ulegała zmianie. Co ciekawe ekspresja mRNA dla Ki-67 ulegała zmniejszeniu w całym miokardium.
6. Ekspresja mRNA dla SRC, FAK, PXN, VE-kadheryny, AKT1, PI3K, eNOS, FOXO1, FOXO3a i PTEN była znacząco podwyższona w komórkach śródbłonka izolowanych z miokardium myszy db/db.
7. Zmniejszenie gęstości ekspresji dla VE-kadheryny na skrawkach z serca myszy db/db w komórkach śródbłonka wykazano jedynie dla lewej komory serca.
8. W teście krążków aortalnych wykazano, że aorty izolowane z myszy db/db w odpowiedzi na stymulację czynnikiem proangiogennym VEGF-A produkują znacząco mniej nowych pączków naczyniowych.

Na podstawie powyższych wyników sformułowano wnioski ogólne:

1. W MetS dochodzi do obniżenia gęstości mikronaczyń w miokardium oraz ich zwiększonej przepuszczalności, co może być efektem zaburzeń w kaskadzie sygnalizacyjnej VEGF/VEGFR
2. Izolacja komórek śródbłonka jest niezbędnym etapem analizy zaburzeń odpowiedzi angiogennej czy przepuszczalności naczyń, gdyż badanie całego miokardium daje wyniki zafałszowane przez obecność innych komórek
3. Obniżona odpowiedź angiogenna śródbłonka w MetS może być wynikiem zaburzeń w kaskadzie sygnalizacyjnej VEGF/VEGFR
4. Konieczne są dalsze prace w szczególności łączące interakcje pomiędzy innymi komórkami, aby dokładnie przedstawić mechanizm w jakim dochodzi do spadku gęstości naczyń i zwiększenia ich przepuszczalności w MetS
5. Wyjaśnienie mechanizmów zaburzających odpowiedź angiogenną i zwiększających przepuszczalność naczyń w sercu w MetS może mieć potencjalne znaczenie praktyczne w profilaktyce, diagnostyce oraz leczeniu niewydolności serca w MetS już na wczesnych etapach tego schorzenia, dlatego dalsze prowadzenie badań w tym zakresie powinno być kontynuowane

8. Oświadczenia wszystkich współautorów publikacji

Warszawa, 05.12.2024
(miejscowość, data)

Mateusz Bartkowiak
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
pomoc przy przygotowaniu literatury oraz korekta artykułu
Mój udział procentowy w przygotowaniu publikacji określam jako 2 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Mateusz Bartkowiak
(podpis oświadczającego)

Warszawa, 1.12.2014
(miejscowość, data)

Ewa Jankowska-Steifer

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
pomoc przy przygotowaniu artykułu oraz jego korekta
Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

(podpis oświadczającego)

Warszawa, 1.12.2024
(miejscowość, data)

Anna Ratajska

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
pomoc przy przygotowaniu artykułu oraz jego korekta
Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Ratajska
(podpis oświadczającego)

Warszawa, 1.12.2024
(miejscowość, data)

Marek Kujawa

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
pomoc przy przygotowaniu artykułu oraz jego korekta
Mój udział procentowy w przygotowaniu publikacji określam jako 4 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Marek Kujawa
(podpis oświadczającego)

Warszawa, 1.12.2014
(miejscowość, data)

Olga Aniołek

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
korekta publikacji
Mój udział procentowy w przygotowaniu publikacji określam jako 1 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

.....
(podpis oświadczającego)

Warszawa, 1.12.2025
(miejscowość, data)

Justyna Niderla-Bielińska

OŚWIADCZENIE

Jako współautor pracy pt. „Metabolic Syndrome and Cardiac Vessel Remodeling Associated with Vessel Rarefaction: A Possible Underlying Mechanism May Result from a Poor Angiogenic Response to Altered VEGF Signaling Pathways." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:
pomoc przy przygotowaniu artykułu oraz jego ostateczna korekta
Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 75 %, obejmował on koncepcję pracy, zebranie literatury, jej analizę oraz przedstawienie w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Justyna Niderla-Bielińska
(podpis oświadczającego)

Warszawa, 05.12.2024
(miejscowość, data)

Mateusz Bartkowiak
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pomoc przy przeprowadzaniu badań oraz analizy statystycznej

Mój udział procentowy w przygotowaniu publikacji określam jako 1 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %,
obejmował on koncepcję pracy, metodologię, przeprowadzenie doświadczeń oraz zebranie literatury, interpretację wyników oraz przedstawienie ich w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Mateusz Bartkowiak
(podpis oświadczającego)

Warszawa, 1.12.2014
(miejscowość, data)

Ewa Jankowska-Steifer

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pomoc przy opracowaniu koncepcji pracy, przeprowadzaniu doświadczeń oraz korektę publikacji

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %,
obejmował on koncepcję pracy, metodologię, przeprowadzenie doświadczeń oraz zebranie literatury, interpretację wyników oraz przedstawienie ich w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

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(podpis oświadczającego)

Warmace, 1.12.2024
(miejscowość, data)

Anna Ratajska

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

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Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Ratajska
(podpis oświadczającego)

Warszawa, 05.12.2024
(miejscowość, data)

Elżbieta Czarnowska

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pomoc przy przygotowaniu koncepcji oraz metodologia i przeprowadzenie doświadczeń z zakresu szczególnie mikroskopii elektronowej.

Mój udział procentowy w przygotowaniu publikacji określam jako 5 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %,
obejmował on koncepcję pracy, metodologię, przeprowadzenie doświadczeń oraz zebranie literatury, interpretację wyników oraz przedstawienie ich w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Elżbieta Czarnowska

(podpis oświadczającego)

Warmia, 1.12.2024
(miejscowość, data)

Marek Kujawa

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pomoc przy opracowaniu koncepcji pracy oraz korektę publikacji

Mój udział procentowy w przygotowaniu publikacji określam jako 3 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %,
obejmował on koncepcję pracy, metodologię, przeprowadzenie doświadczeń oraz zebranie literatury, interpretację wyników oraz przedstawienie ich w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Marek Kujawa
(podpis oświadczającego)

Wolmir 1.12.2024
(miejscowość, data)

Olga Aniołek

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

korekta publikacji

Mój udział procentowy w przygotowaniu publikacji określam jako 1 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %, obejmował on koncepcję pracy, metodologię, przeprowadzenie doświadczeń oraz zebranie literatury, interpretację wyników oraz przedstawienie ich w postaci publikacji.

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

.....
(podpis oświadczającego)

Warszawa, 1.12.2024
(miejscowość, data)

Justyna Niderla-Bielińska

OŚWIADCZENIE

Jako współautor pracy pt. „Expression of mRNA for molecules that regulate angiogenesis, endothelial cell survival, and vascular permeability is altered in endothelial cells isolated from db/db mouse hearts." oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

pomoc przy opracowaniu koncepcji pracy, metodologii, przeprowadzaniu doświadczeń oraz korektę publikacji

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %.

Wkład Krzysztofa Bartkowiaka w powstawanie publikacji określam jako 70 %,
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Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej lek. Krzysztofa Bartkowiaka

Just Nidu-Bieliń
(podpis oświadczającego)

9. Opinia Komisji Bioetycznej lub Etycznej

Zgodnie z ustawą z dnia 15 stycznia 2015 r. *o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych*, z uwagi na charakter wykonywanych prac, nie była wymagana zgoda Komisji Etycznej na przeprowadzenie doświadczeń z wykorzystaniem zwierząt w ramach niniejszej rozprawy doktorskiej.

10. Piśmiennictwo

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