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ROZPRAWA DOKTORSKA

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Wpływ endogennych glikokortykoidów na metabolizm tlenowy i atrofię mięśnia szkieletowego

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DOCTORAL DISSERTATION

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The effect of endogenous glucocorticoids on aerobic metabolism and skeletal muscle atrophy

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SPIS TREŚCI

1. Wykaz stosowanych skrótów (abbreviations)

ATP – (ang. Adenosine triphosphate) – Adenozynotrójfosforan

BST – (ang. Bed nucleus of the stria terminalis) – Jądro łożyskowe prążka krańcowego

CAT – (ang. Catalase) – Katalaza

Cu/ZnSOD – (ang. Copper-zinc superoxide dismutase) – Dysmutaza ponadtlenkowa zawierająca miedź i cynk

DEX – (ang. Dexamethasone) – Deksametazon

EDL – (ang. Extensor digitorum longus muscle) – Mięsień prostownik długi palców

FBXO32 – (ang. F-box only protein 32) – Atrogina-1

GK – (ang. Glucocorticoids) – Glikokortykoidy

GPx – (ang. Glutathione peroxidase) – Peroksydaza glutationowa

GR – (ang. Glucocorticoids receptor) – Receptor glikokortykoidowy

GRE – (ang. Glucocorticoid response elements) – Genomowe elementy odpowiedzi na glikokortykoidy

HSD11B1 – (ang. 11β-hydroxysteroid dehydrogenase type 1) – Dehydrogenaza 11β-hydroksysteroidowa typu pierwszego

HSD11B2 – (ang. 11β-hydroxysteroid dehydrogenase type 2) – Dehydrogenaza 11β-hydroksysteroidowa typu drugiego

IGF-1 – (ang. Insulin – like Growth Factor 1) – Insulinopodobny czynnik wzrostu – 1

KORT – (ang. Corticosterone) – Kortykosteron

MDA – (ang. Malondialdehyde) – Aldehyd malonowy

MnSOD – (ang. Manganese-dependent superoxide dismutase) – Dysmutaza ponadtlenkowa zależna od manganu

MR – (ang. Mineralocorticoid receptor) – Receptor mineralokortykoidowy

NADPH – (ang. Nicotinamide adenine dinucleotide phosphate) – Zredukowana forma dinukleotydu nikotynoamidoadeninowego

PPN – (ang. Hypothalamus – pituitary – adrenal axis) – Oś podwzgórze – przysadka – nadnercza

SOD – (ang. Superoxide dismutase) – Dysmutaza ponadtlenkowa

SOL – (ang. Soleus muscle) – Mięsień płaszczkowaty

TBARS – (ang. Thiobarbituric acid reactive substance) – Reaktywne substancje kwasu tiobarbiturowego

8-izo – (ang. 8 – isoprostanes) – 8-izoprostany

2. Autoreferat w języku polskim

2.1. Wykaz prac wchodzących w skład rozprawy

1. Mateusz Jakub Karnia, Dorota Myślińska, Katarzyna Patrycja Dzik, Damian Józef Flis, Ziemowit Maciej Ciepielewski, Magdalena Podlacha, Jan Jacek Kaczor. ***The electrical stimulation of the bed nucleus of the stria terminalis causes oxidative stress in skeletal muscle of rats***; Oxidative Medicine and Cellular Longevity 2018 May 31;2018:4671213.
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2. Mateusz Jakub Karnia, Dorota Myślińska, Katarzyna Patrycja Dzik, Damian Józef Flis, Magdalena Podlacha, Jan Jacek Kaczor. ***BST stimulation induces atrophy and changes in aerobic energy metabolism in rat skeletal muscles - the biphasic action of endogenous glucocorticoids***; International Journal of Molecular Science 2020 Apr 17;21(8):2787.
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3. Mateusz Jakub Karnia, Daria Korewo, Dorota Myślińska, Ziemowit Maciej Ciepielewski, Monika Puchalska, Klaudia Konieczna – Wolska, Konrad Kowalski, Jan Jacek Kaczor. ***The Positive Impact of Vitamin D on Glucocorticoids-dependent Skeletal Muscle Atrophy***; Nutrients 2021, 13(3), 936.
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2.2. Wprowadzenie

Stres chroniczny jest jednym z najbardziej alarmujących problemów zdrowotnych współczesnych społeczeństw, mimo to, mechanizmy molekularne, odpowiedzialne za jego negatywne skutki wciąż pozostają niedostatecznie wyjaśnione (Jackson 2014). Jednym z lepiej udokumentowanych czynników molekularnych zaangażowanych w rozwój negatywnych skutków stresu uznaje się glikokortykoidy (GK) (Bose i wsp. 2009). Kataboliczne działanie GK jest dobrze znane, a ich destrukcyjna rola w trakcie długotrwałej ekspozycji na ich działanie przejawia się m.in. w mięśniach szkieletowych, gdzie mogą indukować stres oksydacyjny i atrofię mięśni (Oshima i wsp. 2004; Stahn i wsp. 2007; Tang i wsp. 2013).

Jednym z głównych mechanizmów odpowiedzialnych za uwalnianie endogennych GK jest aktywacja osi podwzgórze - przysadka mózgowa - kora nadnerczy (PPN) (Kudielka i Wust 2010). PPN jest krytycznym elementem reakcji organizmu na stres, a jako jeden z istotniejszych modulatorów tej odpowiedzi wskazuje się jądro łożyskowe prążka krańcowego (BST) (Alheid 2003; Choi i wsp. 2008).

W warunkach stresowych, takich jak ekspozycja na niską temperaturę (Eshkevari i wsp. 2015; Garcia-Diaz i wsp. 2015), wysiłek fizyczny lub w trakcie stanów patofizjologicznych takich jak cukrzyca, choroba nowotworowa, głodzenie, oparzenia, depresja (Hu i wsp. 2009), czy wreszcie rozwijający się zespół przetrenowania u sportowców (Ronsen i wsp. 2002; Taverniers i wsp. 2010) poziom krążących GK ulega znacznemu podwyższeniu. A to z kolei powoduje zmniejszenie tempa syntezy białek i nasilenie proteolizy w celu wytworzenia aminokwasów, służących jako prekursor glukoneogenezy w wątrobie. Dodatkowo GK mogą zwiększać wyciek protonów z mitochondriów, wpływając bezpośrednio na aktywność kompleksów białkowych łańcucha oddechowego, potencjał błony mitochondrialnej i syntezę ATP, w konsekwencji zaburzając tlenowy metabolizm energetyczny oraz w skrajnych sytuacjach doprowadzając do śmierci komórki (Stahn, Lowenbergi wsp. 2007). Dlatego też kontrola stężenia kortyzolu może stanowić istotną rolę w procesie monitorowania obciążeń treningowych (Ziemann i wsp. 2012; Balsalobre-Fernandez i wsp. 2014; Backes i wsp. 2015). Ponadto wyniki badań wskazują na dużą przydatność praktyczną parametru jakim jest stosunek insulinopodobny czynnik wzrostu – 1 (IGF-1) do kortyzolu wśród osób trenujących. Autorzy w konkluzji pracy podają, że parametr ten umożliwia trenerowi bieżącą kontrolę obciążeń z uwzględnieniem statusu anaboliczno-katabolicznego organizmu dając możliwość uniknięcia rozwoju stanów ostrego zmęczenia lub

zespołu przetrenowania (Nassib i wsp. 2016). Co więcej dane z piśmiennictwa pokazują, że stężenie kortyzolu może być ściśle związane z wydajnością nerwowo-mięśniową u sportowców najwyższego poziomu (Kraemer i wsp. 2004; Crewther i wsp. 2009).

Coraz więcej wyników badań dowodzi, że efekty wywoływane przez GK mogą być regulowane niezależnie, na wielu poziomach kontroli, przez receptor glikokortykoidowy (GR) i aktywność dehydrogenazy 11β-hydroksysteroidowej typu pierwszego (HSD11B1) zarówno w warunkach *in vitro*, jak i *in vivo*. GK prowadzą swój sygnał głównie przez wewnątrzkomórkowy GR; przypuszczalnie mogą one również działać poprzez szybki mechanizm pozareceptorowy (Kuo i wsp. 2013; Morgan i wsp. 2014).

Główne działanie GR polega na regulacji transkrypcji genów docelowych poprzez przyłączanie do genomowych elementów odpowiedzi na glikokortykoidy (GRE) bezpośrednio wiążąc się z DNA lub poprzez wiązanie z innymi czynnikami transkrypcyjnymi (Kuo i wsp. 2013). Jednak biodostępność i działanie GK zależy nie tylko od ich poziomu w krwioobiegu lub dostępności swoistego receptora, ale także od aktywności specyficznego tkankowo enzymu HSD11B1. Kluczowe metaboliczne tkanki takie jak wątroba, tkanka tłuszczowa czy mięśnie szkieletowe, syntetyzują HSD11B1, której funkcją jest konwersja nieaktywnego kortyzonu do jego aktywnej postaci: kortyzolu lub kortykosteronu (KORT) (Morgan i wsp. 2014). Z opublikowanych wcześniej danych, jasno wynika, że HSD11B1 może być głównym regulatorem atrofii mięśni związanej z działalnością szlaku sygnalizacyjnego AKT-FOXO-Atrogina-1 (Sandri i wsp. 2004). Na przykład zwiększona aktywność HSD11B1 w mięśniach szkieletowych jest związana z rozwojem insulinooporności, spadkiem masy mięśniowej i podwyższoną ekspresją genów związanych z atrofią mięśni (Morgan i wsp. 2009). Odwrotny efekt zaobserwowano natomiast w eksperymencie przeprowadzonym na myszach z nokautem genu dla HSD11B1. Ekspresja genów dla wybranych markerów zaniku mięśni szkieletowych (MuRF-1 i atrogina-1) była niższa u myszy HSD11B1 KO w porównaniu z grupą kontrolną po podaniu KORT (Morgan, McCabe i wsp. 2014).

Ponadto, najnowsze dane wskazują na obecność i aktywność receptora mineralokortykoidowego (MR) w mięśniach szkieletowych [9]. MR i GR wykazują reaktywność krzyżową z endogennymi GK, które mają takie samo lub nawet większe powinowactwo do MR niż GR (w zależności od tkanki). W konsekwencji wysokiej homologii z GR, MR jest aktywowany zarówno przez mineralokortykoidy (aldosteron, deoksykortykosteron), jak i przez GK (Chadwick i wsp. 2015). Dodatkowo, dane z ostatnich kilku lat przypisują MR szczególną rolę pośrednicząco w rozwoju stresu oksydacyjnego.

Wskazują między innymi, że zredukowany fosforan dinukleotydu nikotynoamidoadeninowego (NADPH) zależny od aldosteronu/MR indukuje produkcję ponadtlenków przy jednoczesnym wzroście poziomu 8-izoprostanów (8-izo) oraz reaktywnych substancji kwasu tiobarbiturowego (TBAR) w surowicy (Virdis i wsp. 2002; Gerling i wsp. 2003).

Mimo dużej dostępności danych dotyczących katabolicznej roli długotrwałej ekspozycji na działanie syntetycznych GK (Orzechowski i wsp. 2000; Konno 2005; Jeje i Raji 2015) wciąż niedostatecznie wyjaśniony pozostaje wpływ endogennych GK na metabolizm tlenowy, indukcję stresu oksydacyjnego i atrofię mięśni szkieletowych w trakcie chronicznej reakcji stresowej (CSR). Podjęty w niniejszej pracy problem badawczy wydaje się znaczący, ponieważ jak wskazują dane, egzogenne GK, takie jak deksametazon (DEX), podawane w celu „naśladowania" fizjologicznej reakcji stresowej, mogą nie odzwierciedlać realistycznego stanu obecnego w trakcie nasilonej syntezy endogennych GK (Costantini i wsp. 2011). Istotne podkreślenia są również wyniki badań wskazujące potencjalnie dwukierunkowe (anaboliczne/kataboliczne) działanie GK, w zależności od czasu ekspozycji oraz ich stężenia (Ferretti i wsp. 1992; Goudochnikov 2011). Dlatego też niniejsza praca stanowi próbę oceny wpływu chronicznej reakcji stresowej na metabolizm tlenowy, stres oksydacyjny oraz atrofię w obrębie dwóch typów mięśni szkieletowych o odmiennej charakterystyce histologicznej i metabolicznej (m. płaszczkowaty (SOL) - jako mięsień zbudowany głównie z włókien wolnokurczliwych, o metabolizmie opartym na przemianach tlenowych, oraz m. prostownik długi palców (EDL) – zbudowany głównie z włókien szybkokurczliwych, w którym przeważają procesy beztlenowe).

2.3. Cel pracy i hipoteza

2.3.1. Cel badań

1. Ocena wpływu podwyższonego poziomu endogennych glikokortykoidów na parametry metabolizmu tlenowego oraz stresu oksydacyjnego w mięśniu szkieletowym.
2. Ocena wpływu chronicznej reakcji stresowej na parametry atrofii mięśnia szkieletowego.

2.3.2. Hipotezy badawcze

Podwyższony poziom endogennych glikokortykoidów:

a) zaburza funkcjonowanie metabolizmu tlenowego,

b) indukuje stres oksydacyjny,

c) przyczynia się do atrofii mięśnia szkieletowego.

2.4. Materiały i metody badań

Niniejsza rozprawa doktorska została oparta o cykl trzech prac oryginalnych powiązanych ze sobą tematycznie, opublikowanych w recenzowanych czasopismach naukowych. Wszystkie prace opisują wyniki uzyskane w modelowych warunkach eksperymentalnych chronicznej reakcji na stres, przy czym dwa pierwsze artykuły koncentrują się na reakcji *nieuświadomionej*, indukowanej stymulacją jądra łożyskowego prążka krańcowego (BST). Natomiast trzeci artykuł z cyklu opisuje wyniki uzyskane w modelu chronicznego stresu indukowanego niską temperaturą otoczenia.

Badania zostały przeprowadzone za zgodą Komisji Bioetycznej przy Gdańskim Uniwersytecie Medycznym (nr 8/2010) oraz Komisji Bioetycznej do Spraw Badań na Zwierzętach w Bydgoszczy (nr 12/2019).

W badaniach opublikowanych w pracy nr 1 i 2 wykorzystano 17 szczurów samców szczepu Wistar (8-10 tygodniowe). Zwierzęta po dwutygodniowym okresie aklimatyzacji, zoperowano za pomocą standardowego, stereotaktycznego zabiegu chirurgicznego implementując elektrody stymulacyjne do mózgowia, a następnie przydzielono je losowo do jednej z trzech grup: zwierzęta stymulowane elektrycznie przez dwa tygodnie (ST2; n=6), zwierzęta stymulowane elektrycznie przez cztery tygodnie (ST4; n=6) oraz zwierzęta pozornie stymulowane (SHM; n=5). Krew pobierano dwukrotnie, w narkozie wziewnej, poprzez punkcję serca, pierwszego oraz ostatniego dnia eksperymentu. Następnie pobrana krew została zwirowana przy 2000 × g przez 10 minut w temperaturze 4ºC. Następnie uzyskany materiał biologiczny został podzielony do osobnych probówek, zamrożony i przechowywany w temperaturze -70ºC oczekując na oznaczenia. Po upływie dwóch lub czterech tygodni zwierzęta uśmiercano poprzez podanie dootrzewnowo letalnej dawki pentobarbitalu. Następnie wypreparowywano w całości z dwóch kończyn dolnych mięsień EDL oraz mięsień SOL, które oczyszczano z tłuszczu i tkanki łącznej oraz po umieszczeniu w probówkach do głębokiego mrożenia niezwłocznie zamrażano poprzez umieszczenie pobranego materiału w ciekłym azocie. Przed wykonaniem docelowych oznaczeń biochemicznych mięśnie zostały pocięte, zhomogenizowane i odpowiednio zwirowane. Oznaczenia aktywności enzymów, markerów wolnorodnikowego uszkodzenia białek i lipidów, zawartości poszczególnych białek wykonano w supernatancie mięśni EDL i SOL. Wszystkie metody i oznaczenia zostały zamieszczone w cyklu powiązanych tematycznie publikacji.

W badaniach opublikowanych w ramach pracy nr 3 wykorzystano 45 szczurów samców szczepu Wistar (8-10 tygodniowe). Zwierzęta po dwutygodniowym okresie aklimatyzacji, podzielono losowo na 6 grup: zwierzęta poddawane chronicznemu stresowi suplementowane witaminą D_3 (n=9), zwierzęta poddawane chronicznemu stresowi suplementowane placebo (n=9), zwierzęta poddawane pozorowanemu chronicznemu stresowi (n=6), zwierzęta otrzymujące dootrzewnowo iniekcję syntetycznego GK w postaci deksametazonu (DEX) w dawce 2 mg/kg mc/dzień suplementowane witaminą D_3 (n=8), zwierzęta otrzymujące DEX w dawce 2 mg/kg mc/dzień suplementowane placebo (n=7), oraz zwierzęta z grupy kontrolnej otrzymujące dootrzewnowo iniekcję 0,9% NaCl (n=6). Użyty w eksperymencie model chronicznego stresu stanowił kombinację stresora fizycznego (działanie niskiej temperatury), oraz psychicznego (izolacja oraz niemożność wydostania się z zimnej wody). Przez 28 kolejnych dni, zwierzęta były eksponowane na działanie niskiej temperatury poprzez umieszczenie w klatce o wymiarach 21 × 15 × 30 cm wypełnionej zimną wodą z lodem o głębokości ok 1 cm (0-4ºC) raz dziennie przez 60 minut. Zwierzęta z grupy stresowanej w sposób pozorowany umieszczano natomiast w klatce wypełnionej ciepłą wodą (32-35ºC), raz dziennie przez 60 minut, przez 28 kolejnych dni. Krew pobierano dwukrotnie z żyły ogonowej, w narkozie wziewnej: pierwszego oraz ostatniego dnia eksperymentu. Następnie pobrany materiał był wirowany przy 2000 × g przez 10 minut w temperaturze 4ºC, dzielony do osobnych probówek i zamrażany w -70ºC oczekując na oznaczenia. Ostatniego dnia eksperymentu zwierzęta były uśmiercane poprzez dekapitację, następnie w całości wypreparowywano z dwóch kończyn dolnych, ważono oraz po umieszczeniu w krioprobówkach niezwłocznie zamrażano w ciekłym azocie kolejno: m. EDL, m. SOL, m. piszczelowy przedni, m. brzuchaty łydki, oraz m. czworogłowy uda. Wypreparowano również oraz ważono i po umieszczeniu w krioprobówkach zamrażano w ciekłym azocie: serce, śledzionę, grasicę oraz wątrobę. W ramach oznaczeń biochemicznych w osoczu krwi oznaczono metodą immunoenzymatyczną – *ELISA* stężenie kortykosteronu, oraz metodą chromatografii cieczowej ze spektrometrią mas (LC-MS) stężenie wybranych metabolitów witaminy D_3. Eksperyment składający się na publikację nr 3 został sfinansowany ze środków Narodowego Centrum Nauki w ramach grantu doktoranta Preludium 16 nr 2018/31/N/NZ7/03680.

2.5. Omówienie prac wchodzących w skład rozprawy

W pracy pt. **The electrical stimulation of the bed nucleus of the stria terminalis causes oxidative stress in skeletal muscle of rats** (Autorzy: Karnia M.J., Myślińska D., Dzik K.P., Flis D.J., Ciepielewski Z.M., Podlacha M., Kaczor J.J.; Oxidative Medicine and Cellular Longevity) głównym celem było określenie potencjalnych zmian w zakresie wybranych parametrów stresu oksydacyjnego w dwóch typach mięśnia szkieletowego (EDL i SOL), powodowanych chroniczną stymulacją BST. Wykazano m.in., że czterotygodniowe poddawanie warunkom stresowym poprzez stymulację BST skutkuje aktywacją osi PPN i istotnym statystycznie większym wyrzutem KORT do krwiobiegu w stosunku do zwierząt z grupy kontrolnej. Ponadto, zwiększonego wyrzutu KORT nie zaobserwowano wśród zwierząt poddawanych warunkom stresowym przez dwa tygodnie. W toku badań wykazano różnice w zakresie wolnorodnikowego uszkodzenia białek oraz lipidów w mięśniu białym (EDL) i czerwonym (SOL). Istotny statystycznie wzrost poziomu dwóch markerów wolnorodnikowego uszkodzenia lipidów (aldehydu malonowego – MDA, oraz 8-izoprostanów – 8-izo) obserwowano w grupie stymulowanej przez 4 tygodnie (ST4) w obydwu badanych mięśniach. Niemniej jednak, do wolnorodnikowego uszkadzania białek (mierzonego poziomem grup sulfhydrylowych – grupy SH) dochodziło wyłącznie w mięśniu EDL i to zarówno wśród zwierząt poddawanych stymulacji przez 2 jak i 4 tygodnie. Ponadto, wykazano różnice między mięśniem EDL i SOL w zakresie aktywności całkowitej dysmutazy ponadtlenkowej (SOD), która wzrastała wyłącznie w mięśniu EDL u zwierząt z grupy stymulowanej przez dwa tygodnie (ST2) oraz grupy ST4. Natomiast aktywność mitochondrialnej dysmutazy ponadtlenkowej (MnSOD), katalazy (CAT) oraz peroksydazy glutationowej (GPx) wzrastała zarówno w mięśniu białym jak i czerwonym w grupie ST4. Uzyskane w ramach eksperymentu wyniki dowiodły, że chroniczna ekspozycja na działanie endogennych GK przyczynia się do nasilenia stresu oksydacyjnego w dwóch typach mięśnia szkieletowego, wskazując jednocześnie (oraz potwierdzając wcześniejsze obserwacje innych badaczy) większą wrażliwość mięśnia EDL na chroniczne działanie GK.

W ramach kolejnej pracy z cyklu pt. **BST stimulation induces atrophy and changes in aerobic energy metabolism in rat skeletal muscles - the biphasic action of endogenous glucocorticoids** (Autorzy: Karnia M.J., Myślińska D., Dzik K.P., Flis D.J., Podlacha M., Kaczor J.J.; International Journal of Molecular Science) podjęto próbę wyjaśnienia molekularnego podłoża zachodzących zmian atroficznych w mięśniach szkieletowych oraz

określenia zmiany metabolizmu tlenowego mierzonego aktywnością syntazy cytrynianowej (CS) w mięśniu SOL i EDL. W opublikowanej pracy wykazano, że do istotnego podwyższenia poziomu markera atrofii mięśni – atroginy- 1 (FBXO32) dochodzi zarówno w mięśniu SOL jak i EDL u zwierząt z grupy ST4. Niemniej jednak, w mięśniu SOL obserwowano istotny statystycznie wzrost poziomu IGF-1, stanowiącego prekursor syntezy białek wśród szczurów stymulowanych przez 4 tygodnie. Uzyskany wynik może świadczyć o częściowej anty-atroficznej ochronie mięśnia SOL na działanie GK, co znajduje również odzwierciedlenie w zwiększonej aktywności CS wyłącznie w m. SOL, zbudowanym głównie z włókien wolnokurczliwych, z dużą zawartością mitochondriów. Co więcej, w dyskusji potwierdzono, że wyższa aktywność CS w mięśniu SOL jest konieczna, z uwagi na większe zapotrzebowanie energetyczne mitochondriów, w celu pokrycia rozszerzonego obrotu ATP w trakcie długotrwałej reakcji stresowanej. Wyższa aktywność syntazy cytrynianowej w mięśniu czerwonym mogła być również związana z zwiększonym poziomem iryzyny w surowicy, której zawartość może być zależna od koaktywatora 1-alfa receptora gamma aktywowanego proliferatorami peroksysomów (PGC-1α) w mięśniach szkieletowych, stanowiącego czynnik transkrypcyjny dla biogenezy mitochondriów. Dodatkowo wyniki uzyskane w ramach eksperymentu wskazują, że w warunkach chronicznej reakcji stresowej dochodzi do wysoce istotnego statystycznie wzrostu poziomu MR w mięśniu białym, w którym MR stanowi dominujący „punkt" wiążący krążący KORT (przeciwnie do mięśnia SOL, w którym dominującą rolę receptorową stanowił GR).

Celem 3-ciej pracy cyklu zatytułowanej **The Positive Impact of Vitamin D on Glucocorticoids-dependent Skeletal Muscle Atrophy** (Autorzy: Karnia M.J., Korewo D., Myślińska D., Ciepielewski Z.M., Puchalska M., Konieczna-Wolska K., Kowalski K., Kaczor J.J.) była próba oceny potencjału witaminy D w hamowaniu atrofii mięśniowej indukowanej chroniczną ekspozycją na stres poprzez zanurzenie w zimnej wodzie, oraz atrofii mięśni indukowanej chronicznym podawaniem DEX. Użyty model chronicznej reakcji stresowej skutkował wysoce istotnym statystycznie podniesieniem stężenia KORT w grupie zwierząt stresowanych w stosunku do grupy stresowanej pozornie (zwierzęta zanurzane w ciepłej wodzie). Niemniej jednak, sprzecznie z przyjętą hipotezą, mimo podwyższenia poziomu krążącego KORT nie zaobserwowano zmian w zakresie masy ciała oraz masy poszczególnych mięśni szkieletowych w grupie zwierząt poddawanych chronicznemu stresowi. Częściowym wyjaśnieniem tego fenomenu może być specyfika dobranego stresora w postaci zimnego otoczenia, co mogło skutkować nasileniem lipogenezy z jednoczesnym

brunatnieniem tkanki tłuszczowej. Tego typu obserwację poczyniono w trakcie autopsji zwierząt w punkcie referencyjnym jaki stanowił obszar nadłopatkowy oraz międzyłopatkowy (Zhang i wsp. 2018), w którym depozyt brunatnej tkanki tłuszczowej u zwierząt poddawanych zanurzaniu w zimnej wodzie był zauważalnie większy niż u zwierząt z grupy kontrolnej (wynik nieopublikowany). Niemniej jednak chroniczna ekspozycja na egzogennie podawane GK skutkowała istotnym statystycznie obniżeniem masy ciała oraz masy mięśnia czerwonego i białego. Uzyskane wyniki, wskazujące na dwukierunkowe działanie endo i egzogennych GK, mogą częściowo potwierdzać przypuszczenia wskazane we wstępie niniejszej pracy, jakoby syntetyczne GK podawane w celu „naśladowania" fizjologicznej reakcji stresowej, mogą nie odzwierciedlać stanu obecnego w trakcie faktycznie działającego stresora. Dodatkowo w ramach eksperymentu, wykazano potencjalnie anty-atroficzne działanie witaminy D_3 w zakresie mięśnia SOL. Częściowym wytłumaczeniem tego fenomenu może być zwiększona ekspresja receptora dla witaminy D (VDR) w mięśniu czerwonym niż białym, co skutkuje łatwiejszą biodostępnością witaminy D i jej potencjalnie większą infiltracją w obręb mięśnia czerwonego (Srikuea i wsp. 2020).

2.6. Wnioski

1) Chronicznie podwyższony poziom endogennych glikokortykoidów indukuje stres oksydacyjny w mięśniu szkieletowym, oraz zaburza metabolizm tlenowy, szczególnie w mięśniu białym.
2) Chroniczna reakcja stresowa indukowana stymulacją BST powoduje atrofię mięśniową, szczególnie w mięśniu białym.
3) Chroniczna reakcja stresowa indukowana zimnem aktywuje oś PPN, nie wpływając jednocześnie na masę ciała i masę mięśni szkieletowych.

Wnioski uzyskane w ramach niniejszej pracy potwierdzają obserwacje innych autorów o negatywnej roli stresu (lub ściślej działalności endogennych glikokortykoidów), zarówno w aspekcie zdrowotnym jak i treningowym (Armstrong i VanHeest 2002). Szereg najnowszych wyników badań jasno wskazuje, na aspekt podwyższonej aktywności osi PPN w trakcie rozwijającego się zespołu przetrenowania (Cadegiani i Kater 2019; Cadegiani i Kater 2019; Ushiki i wsp. 2020; Anderson i wsp. 2021). Jednocześnie wskazując na elementy psychoneuroendokrynologiczne jako te, które mogą determinować zwiększoną urazowość mięśniowo-szkieletową u zawodników chronicznie przemęczonych (Smith 2004; de Sousa Nogueira Freitas i wsp. 2020).

3. Dissertation summary

3.1. List of papers included in the dissertation

1. Mateusz Jakub Karnia, Dorota Myślińska, Katarzyna Patrycja Dzik, Damian Józef Flis, Ziemowit Maciej Ciepielewski, Magdalena Podlacha, Jan Jacek Kaczor. ***The electrical stimulation of the bed nucleus of the stria terminalis causes oxidative stress in skeletal muscle of rats***; Oxidative Medicine and Cellular Longevity 2018 May 31;2018:4671213. doi: 10.1155/2018/4671213

Impact Factor: 5.076; Ministerial score: 100

PhD candidate contribution: preparation of a research project, conducting research, statistical analysis, interpretation of results, preparation of publications, preparation of bibliography, obtaining funds

2. Mateusz Jakub Karnia, Dorota Myślińska, Katarzyna Patrycja Dzik, Damian Józef Flis, Magdalena Podlacha, Jan Jacek Kaczor. ***BST stimulation induces atrophy and changes in aerobic energy metabolism in rat skeletal muscles - the biphasic action of endogenous glucocorticoids***; International Journal of Molecular Science 2020 Apr 17;21(8):2787. doi: 10.3390/ijms21082787

Impact Factor: 4.556; Ministerial score: 140

PhD candidate contribution: preparation of a research project, conducting research, statistical analysis, interpretation of results, preparation of publications, preparation of bibliography, obtaining funds

3. Mateusz Jakub Karnia, Daria Korewo, Dorota Myślińska, Ziemowit Maciej Ciepielewski, Monika Puchalska, Klaudia Konieczna – Wolska, Konrad Kowalski, Jan Jacek Kaczor. ***The Positive Impact of Vitamin D on Glucocorticoids-dependent Skeletal Muscle Atrophy***; Nutrients 2021, 13(3), 936. doi: 10.3390/nu13030936

Impact Factor: 4.546; Ministerial score: 140

PhD candidate contribution: preparation of a research project, conducting research, statistical analysis, interpretation of results, preparation of publications, preparation of bibliography, obtaining funds under the National Science Center project Preludium 16 No. 2018/31/N/NZ7/03680

Total publication cycle score: Impact Factor: 14.178; Ministerial score: 380

3.2. Introduction

Chronic stress is one of the most alarming health problems of modern societies, yet the molecular mechanisms responsible for its adverse effects remain insufficiently understood (Jackson 2014). One of the better documented molecular factors involved in developing the adverse effects of stress are glucocorticoids (GK) (Bose et al., 2009). GK's catabolic action is well known, and their destructive role during long-term exposure to them is manifested, among others, in skeletal muscle, where they can induce oxidative stress and muscle atrophy (Oshima et al. 2004; Stahn et al. 2007; Tang et al. 2013).

One of the main mechanisms responsible for releasing endogenous GK's is the activation of the hypothalamic-pituitary-adrenal (PPN) axis (Kudielka and Wust 2010). PPN is a critical component of the body's response to stress. The bed nucleus of the stria terminalis (BST) is indicated as one of the most important modulators of this response (Alheid 2003; Choi et al. 2008). Under stressful conditions, such as exposure to low temperature (Eshkevari et al. 2015; Garcia-Diaz et al. 2015), physical effort or during pathophysiological conditions such as diabetes, cancer, starvation, burns, depression (Hu et al. 2009), and finally, the developing overtraining syndrome in athletes (Ronsen et al. 2002; Taverniers et al. 2010), the level of circulating GK's is significantly increased. Moreover, this, in turn, reduces the rate of protein synthesis and increases proteolysis to produce amino acids that serve as a precursor to gluconeogenesis in the liver. Besides, GK's can increase the leakage of protons from mitochondria, directly affecting the activity of protein complexes of the respiratory chain, the mitochondrial membrane potential, and ATP synthesis, as a consequence disrupting oxygen energy metabolism and, in extreme situations, leading to cell death (Stahn, Lowenberg, et al. 2007). Therefore, the control of cortisol concentration may play an essential role in monitoring training loads (Ziemann et al., 2012; Balsalobre-Fernandez et al., 2014; Backes et al., 2015). Moreover, the research results show that the parameter, which is the ratio of insulin-like growth factor - 1 (IGF-1) to cortisol, is handy in practice among people who train. The authors conclude that this parameter allows the trainer to control loads on an ongoing basis, taking into account the body's anabolic-catabolic status, thus avoiding the development of acute fatigue or overtraining syndrome (Nassib et al., 2016). Moreover, data from the literature indicate that cortisol concentration may be closely related to top-level athletes' neuromuscular performance (Kraemer et al. 2004; Crewther et al. 2009).

A growing body of evidence proves that the effects induced by GK's can be independently regulated at multiple levels of control by the glucocorticoid receptor (GR) and the activity of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) both *in vitro* and *in vivo*. The GK's carry their signal mainly through the intracellular GR; presumably, they may also act through a fast over-receptor mechanism (Kuo et al., 2013; Morgan et al., 2014).

The primary function of GR is to regulate the transcription of target genes by attaching to genomic glucocorticoid response elements (GREs) through direct binding to DNA or binding to other transcription factors (Kuo et al., 2013). However, GK's bioavailability and function depend not only on their level in the bloodstream or on the availability of a specific receptor but also on the activity of the tissue-specific enzyme HSD11B1. Critical metabolic tissues such as the liver, adipose tissue, and skeletal muscles synthesize HSD11B1, the function of which is to convert inactive cortisone into its active form: cortisol or corticosterone (KORT) (Morgan et al. 2014). From previously published data, it is clear that HSD11B1 may be a major regulator of muscle atrophy associated with the AKT-FOXO-Atrogin-1 signaling pathway (Sandri et al. 2004). For example, increased HSD11B1 activity in skeletal muscle has been associated with the development of insulin resistance, decreased muscle mass, and increased gene expression associated with muscle atrophy (Morgan et al., 2009). On the other hand, the opposite effect was observed in an experiment in knockout mice for HSD11B1. Gene expression for selected skeletal muscle atrophy markers (MuRF-1 and atrogin-1) was lower in HSD11B1 KO mice than in the control group after KORT administration (Morgan, McCabe, et al. 2014).

Moreover, the latest data indicate the mineralocorticoid receptor (MR) presence and activity in skeletal muscle [9]. MR and GR show cross-reactivity with endogenous GK's, which have the same or even greater affinity for MR than GR (depending on the tissue). As a consequence of high homology with GR, MR is activated by both mineralocorticoids (aldosterone, deoxycorticosterone) and GK (Chadwick et al., 2015). Data from the last few years assign MR a unique role, mediating the development of oxidative stress. They indicate, i.e., that aldosterone / MR-dependent reduced nicotinamide adenine dinucleotide phosphate (NADPH) induces the production of superoxides with a simultaneous increase in the level of 8-isoprostanes (8-izo) and reactive substances of thiobarbituric acid (TBAR) in the serum (Virdis et al. 2002; Gerling) et al. 2003).

Despite the large availability of data on the catabolic role of long-term exposure to the effects of synthetic GK's (Orzechowski et al. 2000; Konno 2005; Jeje and Raji 2015), the influence of endogenous GK's on oxygen metabolism, induction of oxidative stress, and

skeletal muscle atrophy during chronic stress response is still insufficiently elucidated. The research problem undertaken in this study seems significant because, as shown by the data, exogenous GK's, such as dexamethasone (DEX), administered in order to "mimic" the physiological stress response, they may not reflect the natural state of the increased synthesis of endogenous GKs (Costantini et al. 2011). The results of studies showing the potentially bidirectional (anabolic/catabolic) effect of GK's, depending on the exposure time and their concentration, are also important (Ferretti et al. 1992; Goudochnikov 2011).

Therefore, this study is an attempt to assess the effect of the chronic stress response on oxygen metabolism, oxidative stress, and atrophy within two types of skeletal muscles with different histological and metabolic characteristics (the soleus muscle (SOL) - as a muscle composed mainly of slow-twitch fibers, with metabolism based on oxygen, and, the extensor digitorum longus muscle (EDL) - made mainly of fast-twitch fibers, in which anaerobic processes predominate).

3.3. Purpose of the work and hypothesis

3.3.1. Purpose of research

1. Assessment of the influence of an increased level of endogenous glucocorticoids on the parameters of oxygen metabolism and oxidative stress in skeletal muscle.
2. Assessment of the influence of chronic stress reaction on parameters of skeletal muscle atrophy.

3.3.2. Research hypotheses

Increased level of endogenous glucocorticoids:
a) disturbs the functioning of oxygen metabolism,
b) induces oxidative stress,
c) contributes to skeletal muscle atrophy.

3.4. Materials and research methods

This doctoral dissertation was based on three original papers related to each other, published in peer-reviewed scientific journals. All papers describe the results obtained in the model experimental conditions of the chronic response to stress. The first two papers focus on the *unconscious* response induced by stimulation of the Bed nucleus of the stria terminalis (BST). On the other hand, the third article in the series describes the results obtained in the model of chronic stress induced by low ambient temperature.

The research was conducted with the Bioethics Committee's consent at the Medical University of Gdańsk (No. 8/2010) and the Bioethics Committee for Animal Research in Bydgoszcz (No. 12/2019).

In the studies published in works no. 1 and 2, 17 male Wistar rats (8-10 weeks old) were used. After a two-week acclimatization period, the animals were operated on by standard stereotaxic surgery by implanting electrodes into the brain, and then randomly assigned to one of three groups: electrically stimulated animals for two weeks (ST2; n=6), electrically stimulated animals for four weeks (ST4; n=6) and sham stimulated animals (SHM; n=5). Blood was collected twice, under inhalation anesthesia, by puncture of the heart, on the first and last day of the experiment. The collected blood was then centrifuged at 2000 × g for 10 minutes at 4°C. The obtained biological material was then divided into separate test tubes, frozen and stored at -70°C. Two or four weeks later, the animals were sacrificed by intraperitoneal administration of a lethal dose of pentobarbital. Then, from the two lower limbs whole muscles were excised: extensor digitorum longus (EDL) and soleus muscles (SOL), which were cleaned of fat and connective tissue and immediately frozen by placing the collected material in liquid nitrogen. Before the biochemical measurements the muscles were homogenized. The determination of enzyme activity, markers of free radical damage of proteins and lipids, and the proteins' content were performed in the EDL and SOL muscle supernatants. All methods have been included in a series of thematically related publications.

In the studies published in work no. 3, 45 male Wistar rats (8-10 weeks old) were used. The animals after a two-week acclimatization period were randomized into six groups: chronically stressed rats supplemented with vitamin D_3 (n=9), chronically stressed rats supplemented with placebo (n=9), animals subjected to chronic sham stress (n=6). Then, animals subjected to intraperitoneal injection of synthetic GK in the form of dexamethasone (DEX) at a dose of 2 mg/kg BW/day supplemented with vitamin D_3 (n=8), animals receiving DEX at a dose of 2 mg/kg BW/day supplemented with placebo (n=7), and animals from the

control group receiving an intraperitoneal injection of 0.9% NaCl (n=6). The chronic stress model used in the experiment was a combination of a physical stressor (the effect of low temperature) and a mental stressor (isolation and the inability to escape). For 28 consecutive days, animals were exposed to low temperatures by placing them in a 21 × 15 × 30 cm cage filled with ice-water mixture approximately 1 cm deep (0-4°C) once a day for 60 minutes. The sham-stressed animals were placed in a cage filled with warm water (32-35°C) once a day for 60 minutes for 28 consecutive days. Blood was collected twice from the tail vein under inhalation anesthesia: on the first and last day of the experiment. Subsequently, the collected material was centrifuged at 2000 × g for 10 minutes at 4°C, divided into separate tubes, and frozen at -70°C. On the last day of the experiment, the animals were sacrificed by decapitation, then muscles were excised from two lower limbs, weighed, and immediately frozen in liquid nitrogen, in the following order: EDL, SOL, tibialis anterior, gastrocnemius, and quadriceps femoris. The heart, spleen, thymus, and liver were also excised, weighed, and snap frozen in liquid nitrogen. As part of biochemical determinations, the corticosterone concentration in the blood plasma was determined by the immunoenzymatic method - ELISA, and the concentration of selected metabolites of vitamin D_3 by liquid chromatography with mass spectrometry (LC-MS). The experiment included in publication no. 3 was financed by the National Science Center under the grant of a PhD candidate Preludium 16 no. 2018/31/N/NZ7/03680.

3.5. Discussion of works included in the dissertation

In work entitled **The electrical stimulation of the bed nucleus of the stria terminalis causes oxidative stress in skeletal muscle of rats** (Authors: Karnia MJ, Myślińska D., Dzik KP, Flis DJ, Ciepielewski ZM, Podlacha M., Kaczor JJ; Oxidative Medicine and Cellular Longevity), the main goal was to determine the potential changes in selected parameters of oxidative stress in two types of skeletal muscle (EDL and SOL), caused by chronic BST stimulation. It was shown, among others, that four-week exposure to stress conditions by stimulation of BST results in the activation of the PPN axis and a statistically significant greater KORT surge into the bloodstream compared to animals from the control group. Moreover, an increased KORT release was not observed in animals subjected to two-week stress conditions. The study showed differences in the free radical damage of proteins and lipids in white (EDL) and red (SOL) muscle. A statistically significant increase in the level of two markers of free radical damage of lipids (malondialdehyde - MDA and 8-isoprostanes - 8-izo) was observed in the group stimulated for 4 weeks (ST4) in both examined muscles. Nevertheless, free radical damage of proteins (as measured by the level of sulfhydryl groups – SH groups) occurred only in the EDL muscle, both in animals subjected to stimulation for 2 and 4 weeks. Moreover, differences between the EDL muscle and the SOL muscle were demonstrated in terms of total superoxide dismutase (SOD), the activity of which increased only in the EDL muscle in the two-week stimulated (ST2) and the ST4 groups. In contrast, the activity of mitochondrial superoxide dismutase (MnSOD), catalase (CAT), and glutathione peroxidase (GPx) increased in both white and red muscle in the ST4 group. The obtained results proved that chronic exposure to endogenous GK's contributes to the intensification of oxidative stress in two types of skeletal muscle, simultaneously indicating (and confirming the previous observations of other researchers) greater sensitivity of the EDL muscle to the chronic effects of GKs action.

As a part of another work in the series entitled **BST stimulation induces atrophy and changes in aerobic energy metabolism in rat skeletal muscles - the biphasic action of endogenous glucocorticoids** (Authors: Karnia MJ, Myślińska D., Dzik KP, Flis DJ, Podlacha M., Kaczor JJ; International Journal of Molecular Science) an attempt was made to explain the molecular basis of the atrophic changes occurring in skeletal muscles and to determine the changes in oxygen metabolism measured by citrate synthase (CS) activity in the SOL and EDL muscles. The published study showed that a significant increase in the level of the

muscle atrophy marker - atrogin-1 (FBXO32) occurs in both the SOL and EDL muscles in animals from the ST4 group. Nevertheless, a statistically significant increase in the level of IGF-1, which is a precursor of protein synthesis, among rats stimulated for four weeks, was observed in the SOL muscle. The obtained result may indicate partial anti-atrophic protection of the SOL muscle to GK's action, which is also reflected in the increased activity of CS only in SOL, composed mainly of slow-twitch fibers, richer in mitochondria. Moreover, the discussion confirmed that higher CS activity in the SOL muscle is necessary due to the greater energy demand of the mitochondria to cover the extended turnover of ATP during the chronic stress response. The higher activity of citrate synthase in the red muscle could also be related to the increased level of serum irisin, the content of which may be dependent on the 1-alpha coactivator of the peroxisome proliferator-activated gamma receptor (PGC-1α) in skeletal muscle. Additionally, the results obtained as part of the experiment show that under the conditions of a chronic stress response, there is a statistically significant increase in the level of MR in the white muscle, where MR is the dominant "biding point" the circulating KORT (in contrast to the SOL muscle, where GR played the dominant receptor role).

The aim of the third work of the series, entitled **The Positive Impact of Vitamin D on Glucocorticoids-dependent Skeletal Muscle Atrophy** (Authors: Karnia MJ, Korewo D., Myślińska D., Ciepielewski ZM, Puchalska M., Konieczna-Wolska K., Kowalski K., Kaczor JJ) was an attempt to evaluate the potential of vitamin D in inhibiting muscle atrophy induced by chronic exposure to stress by cold water immersion, and muscle atrophy induced by chronic administration of DEX. The used model of chronic stress response resulted in a highly statistically significant increase in KORT concentration in the group of stressed animals compared to the sham-stressed group (animals immersed in warm water). Nevertheless, contrary to the assumed hypothesis, despite the increase in circulating KORT, we did not observe changes in the body weight and mass of individual skeletal muscles in the group of animals subjected to chronic stress. A partial explanation of this phenomenon may be the specificity of the selected stressor in the form of a cold environment, resulting in the intensification of lipogenesis with the simultaneous browning of adipose tissue. This type of observation was made during the autopsy of the animals at the reference point, which was the suprascapular and interscapular area (Zhang et al. 2018), in which the brown adipose tissue deposit in animals subjected to cold water immersion was noticeably greater than in animals from the control group (data not shown).

Nevertheless, chronic exposure to exogenously administered GK resulted in a statistically significant reduction in body weight and the mass of red and white muscles. The obtained results, indicating a biphasic action of endo and exogenous GK, which may partially confirm the assumptions indicated in the introduction to this paper that synthetic GK administered to mimic a physiological stress response may not reflect the current state during the actual stressor. Additionally, as part of the experiment, the potentially anti-atrophic effect of vitamin D_3 in the SOL muscle was demonstrated. Part of the explanation for this phenomenon may be the greater expression of the vitamin D receptor (VDR) in red muscle than in the white muscle, which results in easier bioavailability of vitamin D and its potentially greater infiltration into the red muscle (Srikuea et al. 2020).

3.6. Conclusions

1) Chronically elevated levels of endogenous glucocorticoids induce oxidative stress in skeletal muscle and disturb oxygen metabolism, especially in white muscle.
2) The chronic stress response induced by BST stimulation causes muscle atrophy, especially in white muscle.
3) The chronic stress response induced by cold water immersion activates the PPN axis without affecting body weight and skeletal muscle mass.

The conclusions obtained in this study confirm other authors' observations about the negative role of stress (or, more precisely, the activity of endogenous glucocorticoids), both in terms of health and training (Armstrong and VanHeest 2002). Several studies indicate the aspect of increased activity of the PPN axis during the developing overtraining syndrome (Cadegiani and Kater 2019; Cadegiani and Kater 2019; Ushiki et al. 2020; Anderson et al. 2021) while pointing to the psychoneuroendocrinological elements as these that may determine the increased musculoskeletal trauma in chronically exhausted athletes (Smith 2004; de Sousa Nogueira Freitas et al. 2020).

4. Piśmiennictwo (References)

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5. Streszczenie pracy w języku polskim

Wprowadzenie: Jednym z lepiej udokumentowanych czynników molekularnych zaangażowanych w rozwój negatywnych skutków stresu uznaje się glikokortykoidy (GK). Kataboliczne działanie syntetycznych GK jest dobrze znane, a ich destrukcyjna rola w trakcie długotrwałej ekspozycji na ich działanie przejawia się m.in. w mięśniach szkieletowych, gdzie mogą indukować stres oksydacyjny i atrofię mięśni. Celem niniejszej pracy była ocena wpływu podwyższonego poziomu endogennych GK na parametry metabolizmu tlenowego, stresu oksydacyjnego oraz atrofii mięśnia szkieletowego. **Materiały i metody:** Eksperyment został przeprowadzony w oparciu o dwa modele chronicznego stresu. W pracy nr 1 i 2 niniejszego cyklu wykorzystano 17 szczurów samców szczepu Wistar (8-10 tygodniowe). Zwierzęta po dwutygodniowym okresie aklimatyzacji, zoperowano za pomocą standardowego, stereotaktycznego zabiegu chirurgicznego implementując elektrody stymulacyjne do mózgowia (jądro łożyskowe prążka krańcowego – BST), a następnie przydzielono je losowo do jednej z trzech grup: zwierzęta stymulowane elektrycznie przez dwa tygodnie (ST2; *n*=6), zwierzęta stymulowane elektrycznie przez cztery tygodnie (ST4; *n*=6) oraz zwierzęta pozornie stymulowane (SHM; *n*=5). Krew pobierano dwukrotnie, w narkozie wziewnej, poprzez punkcję serca, pierwszego oraz ostatniego dnia eksperymentu. Ostatniego dnia eksperymentu zwierzęta uśmiercano i pobierano mięsień płaszczkowaty (SOL) oraz prostownik długi palców (EDL), które następnie homogenizowano, odpowiednio frakcjonowano i, w których dokonywano oznaczeń. W badaniach opublikowanych w ramach pracy nr 3 wykorzystano 45 szczurów samców szczepu Wistar (8-10 tygodniowe). Zwierzęta po dwutygodniowym okresie aklimatyzacji, podzielono losowo na 6 grup: zwierzęta poddawane chronicznemu stresowi (poprzez zanurzenie w zimnej wodzie (0-4ºC), raz dziennie przez 60 minut) suplementowane witaminą D_3 (*n*=9), zwierzęta poddawane chronicznemu stresowi suplementowane placebo (*n*=9), zwierzęta poddawane pozorowanemu chronicznemu stresowi (*n*=6), zwierzęta otrzymujące dootrzewnowo iniekcję syntetycznego GK w postaci deksametazonu (DEX) w dawce 2 mg/kg mc/dzień suplementowane witaminą D_3 (*n*=8), zwierzęta otrzymujące DEX w dawce 2 mg/kg mc/dzień suplementowane placebo (*n*=7), oraz zwierzęta z grupy kontrolnej otrzymujące dootrzewnowo iniekcję 0,9% NaCl (*n*=6). Krew pobierano z żyły ogonowej pierwszego oraz ostatniego dnia eksperymentu. Po 28 dniach zwierzęta uśmiercano, a następnie pobierano w całości mięśnie SOL, EDL, brzuchaty łydki, piszczelowy przedni oraz czworogłowy uda. Pobierano również narządy: wątrobę, serce, śledzionę i grasicę. **Wyniki:** Czterotygodniowa stymulacja BST skutkowała

aktywacją osi PPN i istotnym statystycznie większym wyrzutem KORT do krwiobiegu w stosunku do zwierząt z grupy kontrolnej. Ponadto, zwiększonego wyrzutu KORT nie zaobserwowano wśród zwierząt poddawanych warunkom stresowym przez dwa tygodnie. Wykazano różnice w zakresie wolnorodnikowego uszkodzenia białek oraz lipidów w mięśniu białym (EDL) i czerwonym (SOL). Istotny statystycznie wzrost poziomu dwóch markerów wolnorodnikowego uszkodzenia lipidów (aldehydu malonowego – MDA, oraz 8-izoprostanów – 8-izo) obserwowano w grupie stymulowanej przez 4 tygodnie (ST4) w obydwu badanych mięśniach. Do wolnorodnikowego uszkadzania białek (mierzonego poziomem grup sulfhydrylowych – SH) dochodziło wyłącznie w mięśniu EDL i to zarówno wśród zwierząt poddawanych stymulacji przez 2 jak i 4 tygodnie. Ponadto, wykazano różnice między mięśniem EDL i SOL w zakresie aktywności całkowitej dysmutazy ponadtlenkowej (SOD), która wzrastała wyłącznie w mięśniu EDL u zwierząt z grupy ST2 i ST4. Natomiast aktywność mitochondrialnej dysmutazy ponadtlenkowej (MnSOD), katalazy (CAT) oraz peroksydazy glutationowej (GPx) wzrastała zarówno w mięśniu białym jak i czerwonym w grupie ST4. Dodatkowo wykazano, że do istotnego podwyższenia poziomu markera atrofii mięśni – atroginy- 1 (FBXO32) dochodzi zarówno w mięśniu SOL jak i EDL u zwierząt z grupy ST4. Niemniej jednak, w mięśniu SOL obserwowano istotny statystycznie wzrost poziomu IGF-1, stanowiącego prekursor syntezy białek wśród szczurów stymulowanych przez 4 tygodnie. Co więcej zaobserwowano istotną statystycznie wyższą aktywność syntazy cytrynianowej (CS) w mięśniu SOL w grupie ST4 w stosunku do grupy kontrolnej. Dodatkowo wyniki uzyskane w ramach eksperymentu wskazują, że w warunkach chronicznej reakcji stresowej dochodzi do wysoce istotnego statystycznie wzrostu poziomu receptora mineralokortykoidowego (MR) w mięśniu białym, natomiast w mięśniu SOL do wzrostu poziomu receptora glikokortykoidowego (GR). W modelu chronicznego stresu opartego o zanurzenie w zimnej wodzie stwierdzono wysoce istotny statystycznie wzrost stężenia KORT w grupie zwierząt stresowanych w stosunku do grupy stresowanej pozornie (zwierzęta zanurzane w ciepłej wodzie). Niemniej jednak, mimo podwyższenia poziomu krążącego KORT nie zaobserwowano zmian w zakresie masy ciała oraz masy poszczególnych mięśni szkieletowych w grupie zwierząt poddawanych chronicznemu stresowi. **Wnioski:** Chronicznie podwyższony poziom endogennych glikokortykoidów indukuje stres oksydacyjny w mięśniu szkieletowym, oraz zaburza metabolizm tlenowy, głównie w mięśniu białym, powodując jednocześnie atrofię mięśniową, szczególnie w tym mięśniu. Model chronicznej reakcji stresowej oparty o zanurzenie w zimnej wodzie, aktywuje oś PPN nie wpływając jednocześnie na masę ciała i masę mięśni szkieletowych.

6. Streszczenie pracy w języku angielskim

Introduction: One of the better documented molecular factors involved in the development of the adverse effects of stress is glucocorticoids (GK). The catabolic effect of synthetic GKs is well known. Their destructive role during long-term exposure to them is manifested, among others, in skeletal muscle, where they can induce oxidative stress and muscle atrophy. This study aimed to assess the effect of an increased level of endogenous GK on the parameters of oxygen metabolism, oxidative stress, and skeletal muscle atrophy. **Materials and methods:** The experiment was conducted based on two models of chronic stress. In work no. 1 and 2 of this cycle, 17 male Wistar rats (8-10 weeks old) were used. After a two-week acclimatization period, the animals were operated on by standard stereotaxic surgery by inserting stimulation electrodes into the brain (Bed nucleus of the stria terminals - BST), and then randomly assigned to one of three groups: electrically stimulated animals for two weeks (ST2; *n*=6), electrically stimulated animals for four weeks (ST4; *n*=6) and sham stimulated animals (SHM; *n*=5). Blood was collected twice, under inhalation anesthesia, by puncture of the heart, on the first and last day of the experiment. On the last day of the experiment, animals were sacrificed, soleus muscle (SOL) and extensor digitorum longus muscle (EDL) were harvested, homogenized, and assayed. In the studies published in work No. 3, 45 male Wistar rats (8-10 weeks old) were used. After a two-week acclimatization period, the animals were randomized into six groups: animals subjected to chronic stress (by immersion in cold water (0-4°C), once daily for 60 minutes) supplemented with vitamin D_3 (*n*=9), animals subjected to chronic stress supplemented with placebo (*n*=9), animals subjected to sham chronic stress (*n*=6), animals receiving an intraperitoneal injection of synthetic GK in the form of dexamethasone (DEX) at a dose of 2 mg/kg BW/day supplemented with vitamin D_3 (*n*=8), animals receiving DEX in at a dose of 2 mg/kg BW/day supplemented with placebo (*n*=7), and animals from the control group receiving an intraperitoneal injection of 0.9% NaCl (*n*=6). Blood was collected from the tail vein on the first and last day of the experiment. After 28 days, the animals were sacrificed, and then the whole SOL, EDL, gastrocnemius, tibialis anterior, and quadriceps femoris muscles were excised. The following organs were also collected: liver, heart, spleen, and thymus. **Results:** Four-week BST stimulation resulted in the activation of the PPN axis and statistically significant higher KORT surge into the bloodstream compared to the animals from the control group. Moreover, an increased KORT release was not observed in animals subjected to the ST2 group. Differences in free radical damage of proteins and lipids markers in white (EDL) and red (SOL) muscle were found. A statistically significant increase in the

level of two markers of free radical damage of lipids (malondialdehyde - MDA and 8-isoprostanes - 8-iso) was observed in the group stimulated for four weeks (ST4) in both examined muscles. Free radical damage of proteins (measured by the level of sulfhydryl groups - SH) occurred only in the EDL muscle. Moreover, differences were shown between EDL and SOL in total superoxide dismutase (SOD) activity, which only increased in EDL muscle in the ST2 and the ST4 groups. In contrast, the activity of mitochondrial superoxide dismutase (MnSOD), catalase (CAT), and glutathione peroxidase (GPx) increased in both white and red muscle in the ST4 group. Also, it has been shown that a significant increase in the level of the muscle atrophy marker - atrogin-1 (FBXO32) occurs in both the SOL and EDL muscles in animals from the ST4 group. Nevertheless, a statistically significant increase in the level of IGF-1, which is a precursor of protein synthesis, in rats stimulated for four weeks, was observed in the SOL muscle. Moreover, statistically significant higher activity of citrate synthase (CS) in the SOL muscle was observed in the ST4 group compared to the control group. In addition, the results obtained in the experiment show that under the conditions of the chronic stress response, there is a statistically significant increase in the level of the mineralocorticoid receptor (MR) in the white muscle. In contrast, in the SOL muscle, there is an increase in the level of the glucocorticoid receptor (GR). In the model of chronic stress based on cold water immersion, a statistically significant increase in KORT concentration was found in the group of stressed animals, contrary to the sham-stressed group (animals immersed in warm water). Nevertheless, despite the increase in the circulating level of KORT, no changes in body weight and the mass of individual skeletal muscles were observed in the group of animals subjected to chronic stress. **Conclusions:** Chronically elevated levels of endogenous glucocorticoids induce oxidative stress in skeletal muscle and disturb oxygen metabolism, especially in white muscle, while causing muscle atrophy, especially in white muscle. The chronic stress response model based on cold water immersion activates the PPN axis without affecting the body weight and skeletal muscle mass.

7. Załączniki

- Oświadczenia współautorów,
- Publikacja nr 1. *The electrical stimulation of the bed nucleus of the stria terminalis causes oxidative stress in skeletal muscle of rats*
- Publikacja nr 2. *BST stimulation induces atrophy and changes in aerobic energy metabolism in rat skeletal muscles - the biphasic action of endogenous glucocorticoids*
- Publikacja nr 3. *The Positive Impact of Vitamin D on Glucocorticoids-dependent Skeletal Muscle Atrophy*

SZKOŁA DOKTORSKA

OŚWIADCZENIE WSPÓŁAUTORÓW PUBLIKACJI

Karnia M.J., Myślińska D., Dzik K.P., Flis D.J., Ciepielewski Z.M., Podlacha M., Kaczor J.J., (2018). **The Electrical Stimulation of the Bed Nucleus of the Stria Terminalis Causes Oxidative Stress in Skeletal Muscle of Rats**. *Oxidative Medicine and Cellular Longevity, Volume 2018*, Article ID 4671213. https://doi.org/10.1155/2018/4671213

Niniejszym oświadczamy, że indywidualny wkład w powstanie ww publikacji jest następujący:

autor	wkład %	opis*	podpis
Mateusz Jakub Karnia	50	A, B, C, D, E, F, G	
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Magdalena Podlacha	5	B	
Jan Jacek Kaczor	15	A, D, E, G	

* **A** – przygotowanie projektu badania, **B** – przeprowadzanie badań, **C** – analiza statystyczna, **D** – interpretacja wyników, **E** – przygotowanie publikacji, **F** – opracowanie piśmiennictwa, **G** – pozyskanie funduszy

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Flis Damian Józef	5	B	
Magdalena Podlacha	5	B	
Jan Jacek Kaczor	20	A, D, E, G	

* **A** – przygotowanie projektu badania, **B** – przeprowadzanie badań, **C** – analiza statystyczna, **D** – interpretacja wyników, **E** – przygotowanie publikacji, **F** – opracowanie piśmiennictwa, **G** – pozyskanie funduszy

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Dorota Myślińska	8	B	
Ziemowit Maciej Ciepielewski	3	B	
Monika Puchalska	3	B	
Klaudia Konieczna-Wolska	3	B	
Konrad Kowalski	3	D	
Jan Jacek Kaczor	10	A, D, E	

* **A** – przygotowanie projektu badania, **B** – przeprowadzanie badań, **C** – analiza statystyczna, **D** – interpretacja wyników, **E** – przygotowanie publikacji, **F** – opracowanie piśmiennictwa, **G** – pozyskanie funduszy

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Research Article

The Electrical Stimulation of the Bed Nucleus of the Stria Terminalis Causes Oxidative Stress in Skeletal Muscle of Rats

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Recent studies indicate that activation of hypothalamus-pituitary-adrenocortical axis (HPA) plays the crucial role in stress response, while several lines of evidence mark the bed nucleus of the stria terminalis (BST) as a major mediator of the HPA axis responses to stress. The purpose of this study was to investigate the influence of the corticosterone flux induced by the electrical stimulation of BST on markers of free radical damage of lipids and proteins and antioxidant enzyme activity in skeletal muscle of rats. The male Wistar rats were used and assigned to one of three groups: sham-operated (SHM; $n = 6$), two-week (ST2; $n = 6$), and four-week stimulated (ST4; $n = 5$) groups. Blood, soleus, and extensor digitorum longus muscles were collected. The chronic, 4-week electrical stimulation of the BST evokes increased plasma corticosterone concentration, which resulted in oxidative stress in skeletal muscles. We found higher level of lipid peroxidation markers, lower level of protein oxidation marker, and elevated antioxidant enzyme activity in both muscles. Our findings have also potential implication showing that reaction to the long-term "psychological stress" may lead to free radical damage of muscle.

1. Introduction

It is well known that chronic stress response has extremely negative consequences for health. However, the molecular mechanisms that trigger and accelerate the process of stress inducing life-threatening effects, death, and the pathophysiological complications of diseases that arise from stress has not been definitively clarified. A number of studies have focused on the understanding of molecular pathways that determine system response to stress through the release of glucocorticoids (GCs) [1, 2]. The catabolic effects of GCs are well known, and destructive role of GCs is manifested in skeletal muscle disorders, including oxidative stress and muscle atrophy [3–8].

The oxidative stress is defined as a disturbance of prooxidant-antioxidant balance in the direction of oxidation reaction. This stress is a series of biochemical reactions that result in free radical cell damage or its compartments by reactive oxygen and nitrogen species (RONS). Mitochondria and cytoplasm are the main sources of reactive oxygen species (ROS) generation in the skeletal muscle [3]. Recently, it has been shown that both sources of ROS generation are involved in aging process and dysfunction in several metabolic and neurodegenerative diseases, which may be partially connected with elevated GC level [9]. A growing body of evidence demonstrates that GC signaling is a common mediator of wasting, irrespective of the underlying initiator or disease state. The GCs may increase a leakage of protons

from mitochondria, thus affecting the electron transport chain activity, mitochondrial membrane potential, and ATP generation, and may lead to cell injury and cell death [4]. Furthermore, high plasma levels of GCs can alter the physicochemical properties of a biological membrane, particularly cellular and mitochondrial membranes. Recently, it has been presented that GCs may penetrate into the membrane and change functions of membrane proteins, thereby affecting lipid peroxidation and membrane permeability [5], protein carbonylation, and mitochondrial dysfunction [6, 7]. Many previous data have reported the inductive role of GCs on the oxidative stress in the variety of different tissues such as the brain [10–13], liver [11–14], heart [11], bone [9], tendons [15], and muscles [8, 16]. Moreover, disturbances in oxygen metabolism and downregulation of cell's antioxidant capacity are considered to be an important factor in the development of neurodegenerative disorders such as Alzheimer disease (AD) and Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) [17]. In addition, Sato and coworkers have indicated the direct link between the increased level of GCs with ROS generation and the progression of the neurodegenerative diseases [10].

It is generally known that one of the main mechanisms responsible for the release of GCs is hypothalamus-pituitary-adrenocortical axis (HPA) activation [18]. HPA is a critical component of the body's stress response. There is an evidence indicating the bed nucleus of the stria terminalis (BST) to be a major mediator of the HPA axis in the response to stress [19, 20]. Moreover, some data suggest that the BST acts as a relay between limbic processing of emotional information with a final response of the HPA axis [21]. The possible connections act mainly through sending projections from the BST to the corticotrophin-releasing hormone regions of the paraventricular hypothalamic nucleus [22].

Despite the fact that oxidative stress in skeletal muscles has been shown to be induced by GCs, there are no reports regarding the influence of chronic stress response to endogenous GC action on oxidative stress in skeletal muscles through the direct HPA axis activation. Additionally, some previous reports suggest that treatment with exogenous GCs to simulate a stress response may not reflect real state of stress response. The purpose of this study was to determine the chronically persistent endogenous GC release (chronic stress response) induced by the electrical stimulation of the BST on markers of free radical damage of lipids and protein and antioxidant enzyme activity in two different types of the skeletal muscles in rat. We believe that advantage of our model is that it mimics unconscious stress state in stimulated rats [23, 24]. We hypothesize that chronic stress response (CSR) linking with GC action may influence on the oxidative damage in the skeletal muscle in rat.

2. Materials and Methods

2.1. Animals. Male *Wistar* rats (250–300 g) were used. The animals were provided a standard diet *ad libitum* with free access to water and were maintained on a 12 h light/dark cycle, temperature 22°C, and humidity 50–55%. The animals were habituated daily for about two weeks before the experiment to minimize stress caused by the experimental procedures. In brief, the habituation were carried out in a sound attenuating chamber in a $250 \times 350 \times 440$ mm testing box. The rats were taken from their home cages and placed in the testing box where they had free access to food and were allowed to explore the box for 30 min. Experimental procedures were performed between 8 : 00 and 13 : 00. The rats were divided randomly into three groups: the BST two-week electrically stimulated group (ST2; $n = 6$), the BST four-week electrically stimulated group (ST4; $n = 5$), and the BST sham group (SHM; operated but not stimulated; $n = 6$).

The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC), and the protocols were approved by the Local Animal Research Ethical Committee for the Care and Use of Laboratory Animals at the Medical University in Gdansk, Poland (number 8/2010).

2.2. Surgery. Standard stereotaxic surgery was performed under pentobarbital anesthesia (60 mg/kg i.p.) (Vetbutal, Biowet Puławy, Poland) with a premedication of xylazine (5 mg/kg i.p.) (Sedazin, Biowet, Puławy) and 0.1% solution of atropine (0.25 mg per animal) according to Myślińska and coworkers [25].

2.3. The Electrical Stimulation. The electrical stimulation was performed following the procedure described before [25]. Briefly, two weeks after recovery from the surgery, the stimulated groups were screened for BST stimulation-induced behavior. Once determined, the current intensity was held constant throughout 14 (ST2) or 28 (ST4) consecutive days of the stimulation. The animals from the SHM group were treated in the same way as the experimental group with the exception that no current was passed through the electrodes.

2.4. Animal Sacrifice, Blood, and Muscle Collection. All animals were sacrificed at required time point (one hour after termination of the last electric stimulation). The blood samples were collected by heart puncture one hour after the last session of stimulation. The blood was centrifuged at $2000g$ for 10 min at 4°C. Plasma was separated and stored at −80°C for later analysis. Extensor digitorum longus (EDL) and soleus (SOL) muscles were removed from both hind limbs, dissected from fat and connective tissue, and placed into individual tubes for immediate freezing in liquid nitrogen. All samples were stored at −80°C until analysis. After sacrificing, rats' brains had perfused by 4% polyformaldehyde and removed in order to carry out histological verification of the electrodes' placement.

2.5. Muscle Homogenization. Prior to the chemical assays, muscles were minced and homogenized in an ice-cold buffer that contained 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, 1.15% KCl, and 1 : 200 protease inhibitor (Sigma-Aldrich, P8340) at pH 7.4. The homogenates were then centrifuged at $750g$ at 4°C for 10 min, then supernatants were collected and stored in −80°C until analysis. Protein concentration was determined using the

Bradford assay (Sigma-Aldrich, B6916) according to manufacturer's instructions.

2.6. Plasma Corticosterone (CORT). The plasma CORT concentration was determined by radioimmunoassay using a commercially available kit (Rat corticosterone 125I RIA kit, Institute of Isotopes, Budapest, Hungary) and Wizard 1470 gamma counter. The concentration of CORT was expressed as nanogram per ml of plasma.

2.7. Enzyme Activities and Oxidative Stress Markers in Skeletal Muscles

2.7.1. Superoxide Dismutase (SOD). The muscle SOD activity was determined in the SOL and EDL muscles by measuring the kinetic consumption of $O_2^{-\bullet}$ by superoxide dismutase in a competitive reaction with cytochrome c, as described by Flohé and Ötting [26]. In the SOL muscle, 7.5 μl of supernatant was added to a cuvette containing 982.5 μl of medium (50 mM phosphate buffer, 1 mM EDTA, pH 7.8, with partially acetylated cytochrome c (25 mg/100 ml)) and xanthine (0.5 μM). In the EDL muscle, 15 μl of supernatant and 975 μl of medium were added. 10 μl of xanthine oxidase (0.2 U/ml) was added to initiate the reaction, and absorption was measured at 550 nm for 3 min at 30°C. In a separate cuvette, MnSOD activity was measured on the same sample analyzed under identical conditions with the addition of 10 μl of 200 mM KCN (prepared fresh daily at pH 8.5–9.5). Cu/ZnSOD activity was calculated by subtracting MnSOD activity from total SOD activity. The SOD activities were expressed as units per milligram of protein.

2.7.2. Catalase (CAT). The muscle CAT activity was measured in the SOL and EDL muscles by estimating the kinetic decomposition of H_2O_2, according to Aebi [27]. Briefly, 30 μl of supernatant of the SOL or 100 μl of the EDL from the 750g spin was added to a cuvette containing 960 μl or 890 μl of medium, respectively (50 mM phosphate buffer, 5 mM EDTA, and 0.05% Triton X-100 at pH 7.4). 10 μl of 1 M H_2O_2 was added to the cuvette and mixed to initiate the reaction. Absorbance was measured at 240 nm for 1 min at 30°C. CAT activity was expressed as micromoles per minute per milligram of protein.

All of the samples were analyzed in duplicate, and all kinetics were measured in a temperature-controlled Cecil Super Aquarius CE 9200 spectrophotometer.

2.7.3. Glutathione Peroxidase (GPx). The GPx activity in muscle homogenates was determined by Glutathione Peroxidase Kit (703102, Cayman Chemicals, USA) in accordance with supplied manufacturer's instruction.

The activity of GPx was expressed as nanomoles per minute per milligram of protein.

2.7.4. 8-Isoprostanes (8-iso). A marker of lipid peroxidation, skeletal muscle 8-iso content, was determined with 8-Isoprostane ELISA Kit (516351, Cayman Chemicals, USA) according to the manufacturer's instruction. The concentration of 8-iso was expressed as picograms per milligram of protein.

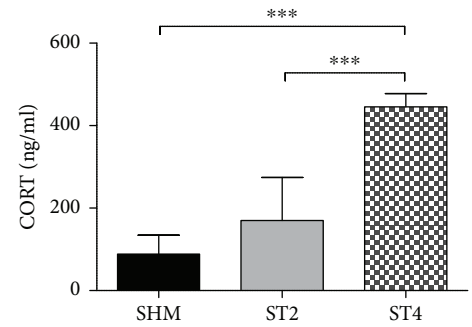

Figure 1: The level of CORT in plasma. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{***}p < 0.001$.

2.7.5. Glutathione Disulfide (GSSG). GSSG was determined by using a kinetic assay as described by Akerboom and Sies [28]. In brief, the homogenate was centrifuged at 5000g for 5 min, the supernatant was neutralized in 300 mM 3-(N-morpholino) propanesulfonic acid in 2 M solution of KOH. The samples were analyzed in the medium containing 100 mM potassium phosphate buffer pH 7.0, 1 mM EDTA, 0.1 mM 5.5′-dithio-bis (2-nitrobenzoic acid) (DTNB), 0.2 U/ml glutathione reductase, and 0.2 mM NADPH and were measured by using the spectrophotometer (Cecil Super Aquarius CE 9200) at 412 nm. A standard curve made of fresh GSSG was used to calculate the concentrations of GSSG. The concentration of GSSG was expressed as nanomoles per milligram of protein.

2.7.6. Sulfhydryl Group Content (SH Groups). The SH group content in the muscle homogenates was measured spectrophotometrically (Cecil Super Aquarius CE 9200) with DTNB assay according to a previously described procedure [29]. Briefly, samples were incubated with 0.1 mM DTNB at room temperature for 60 min. Absorbance was determined at 412 nm. The level of SH groups was expressed as micromoles per gram of tissue.

2.7.7. Malondialdehyde (MDA). The muscle MDA level was determined as previously described [30]. In brief, the absorbance of the 750g centrifuged homogenate was measured on the spectrophotometer (Cecil Super Aquarius CE 9200) at 586 nm. The level of MDA in the samples was determined using 10 mM 1,1,3,3-tetramethoxypropane as a standard. The level of MDA was expressed as micromoles per gram of tissue.

2.7.8. Statistical Analysis. Statistical analysis was performed using a software package (Statistica v. 12.0, StatSoft Inc., Tulsa, OK, USA). The results are expressed as mean ± SD. The differences between groups were tested using one-way ANOVA followed by Tukey post hoc test; p values less than 0.05 were considered statistically significant.

3. Results

3.1. Serum CORT Level. Serum CORT concentration after BST stimulation was significantly higher in the ST4 group as compared with the ST2 and SHM groups ($p < 0.001$; Figure 1). The CORT concentration was 445.9 ± 31.6,

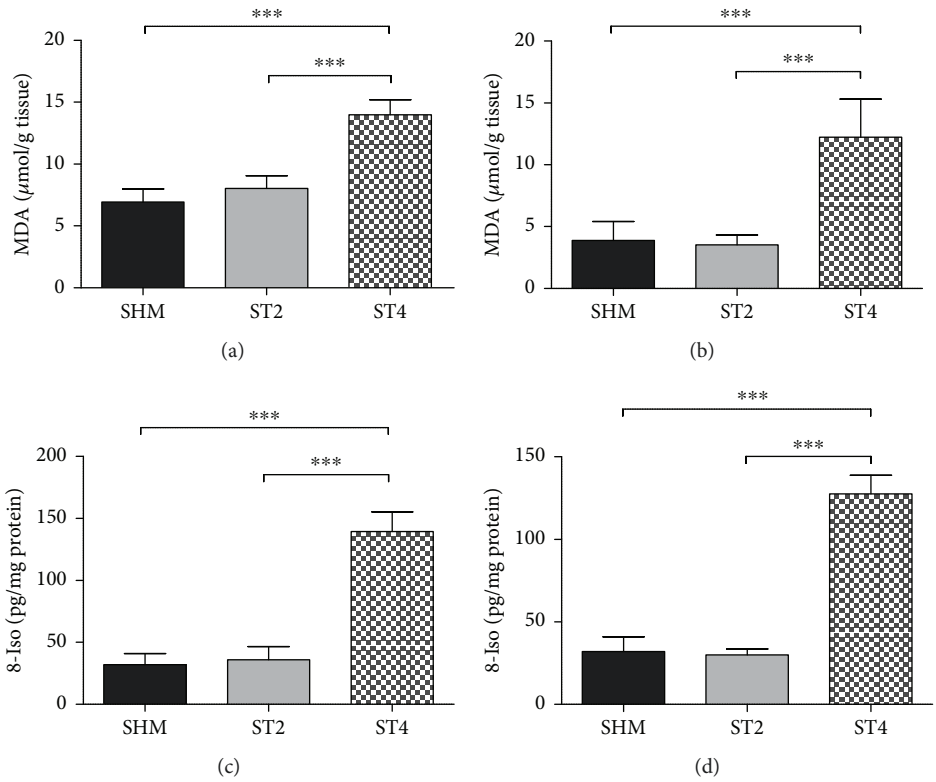

Figure 2: The level of MDA in (a) SOL and (b) EDL. The level of 8-iso in (c) SOL and (d) EDL. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{***}p < 0.001$.

170.1 ± 104.1, and 88.6 ± 45.6 ng/ml in the ST4, ST2, and SHM groups, respectively.

3.2. Oxidative Stress Markers and Enzyme Activities in Skeletal Muscles

3.2.1. Markers of Lipid Peroxidation. The level of both lipid peroxidation markers (MDA and 8-iso) in the SOL and EDL muscles were significantly elevated in the ST4 group compared with the other groups. The concentration of MDA was significantly higher in the ST4 (14.0 μmol/g tissue) than ST2 and SHM groups in the SOL muscle (8.0, $p < 0.001$ and 6.9 μmol/g tissue, $p < 0.001$, resp.) (Figure 2(a)). After 4 weeks of the BST electrical stimulation, the concentration of MDA was also elevated in the ST4 group as compared to both ST2 and SHM groups in the EDL muscle (3.5-fold, $p < 0.001$ and 3-fold, $p < 0.001$, resp.); (Figure 2(b)). The level of 8-iso in the SOL muscle was the highest in the ST4 group (139.4 ± 16.0) as compared with the ST2 (35.8 ± 10.7) and the SHM groups (32.0 ± 8.9 pg/mg protein) ($p < 0.001$; Figure 2(c)). Also, in the EDL muscle, the concentration of 8-iso was elevated approximately fourfold in the ST4 group as compared to the SHM and ST2 groups ($p < 0.001$ Figure 2(d)).

3.2.2. Marker of Protein Oxidation. The level of SH group was not significantly different between groups in SOL muscle after the BST stimulation (Figure 3(a)) and it was 1321.2 in the ST4, 1363.1 in the ST2, and 1378.2 μmol/g tissue in the SHM group, respectively. However, in the EDL muscle, the level of SH groups in the ST4 rats was significantly lower than in the SHM group, also differences in the ST4 as compared to the ST2 group was observed in the EDL muscle after the electrical stimulation ($p < 0.001$; Figure 3(b)). The values of SH groups were 1323.5 ± 62.1, 1476.9 ± 48.7, and 1543.3 ± 41.8 μmol/g tissue in the ST4, ST2, and SHM groups, respectively.

The concentration of GSSG was the highest in the ST4 group in both muscles. In the SOL muscles, there were 500.9 ± 43.8 in the ST4, 395.4 ± 13.8 in the ST2, and 413.9 ± 43.5 nmol/mg protein in the SHM groups, respectively ($p < 0.001$ ST4 versus ST2; $p < 0.01$ ST4 versus SHM) (Figure 4(a)). There was significantly higher level of GSSG in both the ST4 and ST2 in the EDL muscle, as compared to the SHM group ($p < 0.001$ ST4 versus SHM $p < 0.01$ ST2 versus SHM). The values were 219.8 ± 12.9, 200.6 ± 22.1, and 163.0 ± 16.9 nmol/mg protein in the ST4, ST2, and SHM groups, respectively (Figure 4(b)).

3.2.3. Antioxidant Enzyme Activity. The activity of total SOD was higher in the ST4 group compared to the SHM group only in the EDL muscle ($p < 0.001$; Figure 5(b)). Total SOD activity was also elevated in the ST2 group versus the SHM group in the EDL muscle ($p < 0.05$; Figure 5(b)). The activity of total SOD was not different in the SOL muscle after rat stimulation (Figure 5(a)). In Cu/Zn SOD activity, there were no differences between groups in SOL muscle (Figure 5(c)). However, the EDL muscle activity of Cu/ZnSOD was higher

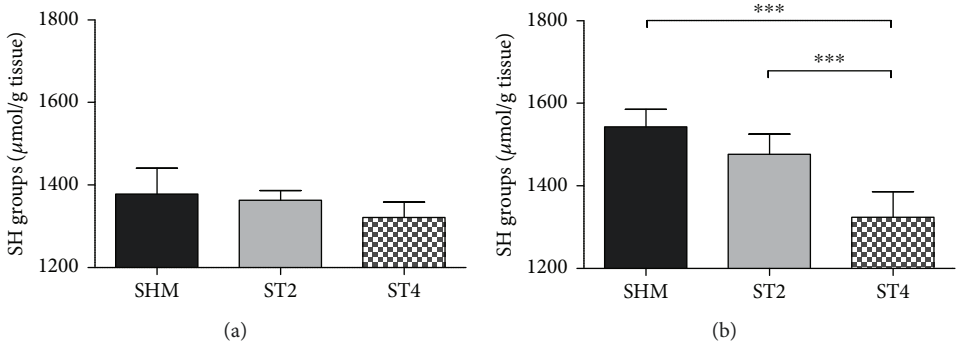

Figure 3: The level of SH groups in (a) SOL and (b) EDL. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{***}p < 0.001$.

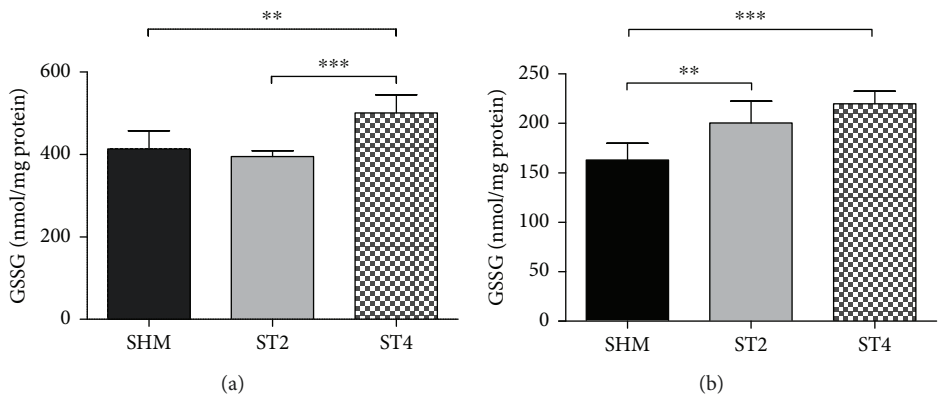

Figure 4: The level of GSSG in (a) SOL and (b) EDL. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{**}p < 0.01$, $^{***}p < 0.001$.

in the ST4 as compared to the SHM group ($p < 0.05$; Figure 5(d)). The mitochondrial isoform of SOD activity was higher in the ST4 group than in both ST2 and SHM groups in the SOL muscle ($p < 0.01$ and $p < 0.05$, resp.; Figure 5(e)). There was also elevated MnSOD activity in the ST4 group versus both the ST2 and SHM groups in EDL muscle ($p < 0.05$; Figure 5(f)).

After 4 weeks of rat stimulation, CAT activity was higher in the ST4 group when compared to the ST2 and SHM groups in both muscles. In the SOL muscle, the activity of CAT was 8.4 ± 0.9 in the SHM group, 8.9 ± 1.9 in the ST2 group, and 13.2 ± 2.3 μmol/min/mg of protein in the ST4 group ($p < 0.01$; ST4 versus ST2 and ST4 versus SHM) (Figure 6(a)). The activity of CAT in the EDL muscle was lower than in the SOL muscle; nevertheless, differences between groups were also significant ($p < 0.01$ ST4 versus ST2, $p < 0.001$ ST4 versus SHM). The CAT activity was 1.4 ± 0.2 in the SHM group, 2.0 ± 0.3 in the ST2 group, and 3.5 ± 1.3 μmol/min/mg of protein in the ST4 group in the EDL muscle after the electrical stimulation of the BST (Figure 6(b)).

GPx activity was significantly higher in the ST4 group compared to the SHM group, both in the SOL and EDL muscles. In the SOL muscle, the value was 13.4 ± 1.3 in the ST4 group, 9.2 ± 0.7 in the ST2 group, and 9.5 ± 0.5 nmol/min/mg protein in the SHM group ($p < 0.001$ ST4 versus ST2 and ST4 versus SHM; Figure 6(c)). In the EDL muscle, significant differences were observed between the ST4 and the SHM groups ($p < 0.01$; Figure 6(d)) and between the ST2 and the SHM groups ($p < 0.05$). The average activity of GPx was 1.6 ± 0.2, 1.5 ± 0.3, and 1.1 ± 0.1 nmol/min/mg of protein in the ST4, ST2, and SHM groups, respectively.

4. Discussion

To the best of our knowledge, this is the first preclinical study showing that the 4-week electrical stimulation of the BST considerably induces HPA axis activation and evokes CORT secretion. In addition, the elevated plasma CORT concentration was associated with increased oxidative stress in skeletal muscle. We found higher concentration of markers of lipid and protein peroxidation and elevated activity of antioxidant enzymes in both SOL and EDL muscles after 4 weeks of stimulation. Moreover, we did not observe similar changes after 2 weeks of stimulation, short stress response (SSR). This observation is consistent with earlier data conducted by Myślińska and coworkers, where a lack of differences was observed in plasma CORT level between the sham-operated and 2-week BST-stimulated groups [25]. Taking into account anatomical and functional basis of the inductive role of the BST in HPA axis activation [19, 20] with simultaneous no differences in plasma CORT level after 2 weeks of the BST

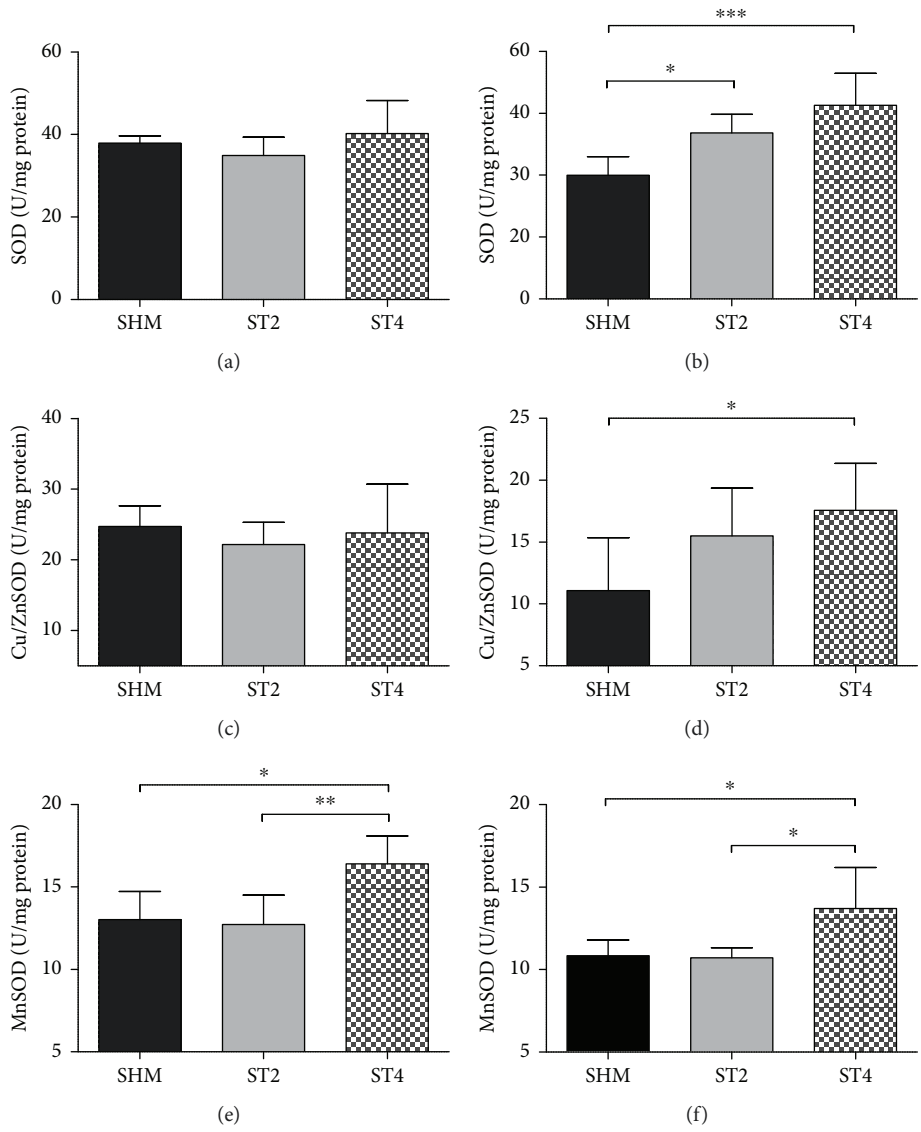

Figure 5: The activity of total SOD in (a) SOL and (b) EDL, Cu/ZnSOD in (c) SOL and (d) EDL, and MnSOD in (e) SOL and (f) EDL. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

stimulation in experimental condition, we decided to extend the time of the electrical stimulation of this limbic structure. Our data suggest that exposure to the CSR (4 weeks of stimulation) is associated with free radical damage of macromolecules in skeletal muscle. In the present study, we noticed fivefold increase of the CORT level in the ST4 group versus the SHM group. It may prove the essential role of the BST in the modulation of CSR, what was widely discussed before [19, 21]. Moreover, obtained data suggest that the chronic 4-week electrical stimulation of BST could mimic long-term mental stress and be useful as a "model" of unconscious stress. In the study by Fontella and coworkers, it was demonstrated that repeated restraint stress entails the increase of plasma CORT level [13]. What is more, another study showed two times higher (compared with [13] and the current study) release of the CORT after restraint stress [12]. Despite the differences in particular data, the multifold increase of plasma CORT level compared with the control group seems to confirm credibility of adopted model of stress response.

4.1. The Exogenous GC Administration as a Mimic of Stress Response. One of the considered methods used to mimic the stress response is an exogenous CORT or other synthetic GC administration. Oshima and coworkers showed that treatment with dexamethasone (DEX) leads to an increased generation of ROS in both human rhabdomyosarcoma and a dopaminergic neuroblastoma cell lines [6]. Also, it was reported that DEX treatment rats decreased GSH content in blood and SOL muscle [16] and GSH/GSSG ratio decreased in the brain in TDP-25 transgenic mice [31]. In the current study, similar effect was observed, where the level of GSSG in both types of muscle was the highest in the ST4 group. Likewise, it was reported that lipid peroxidation markers, TBARs or MDA, were increased in blood [16], brain [32, 33], lymphoid organs [34], and even

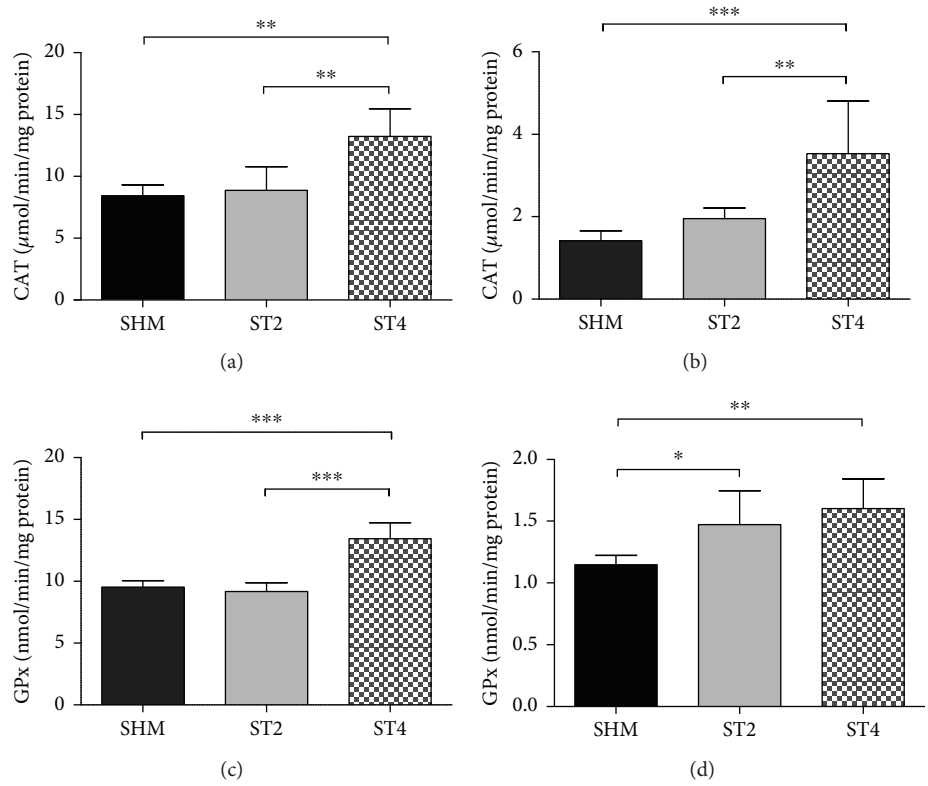

Figure 6: The activity of CAT in (a) SOL and (b) EDL. The activity of GPx in (c) SOL and (d) EDL. Results were expressed as mean ± SD, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

seminal plasma [35] after DEX administration. In the experiment conducted by Pereira and coworkers, higher concentration of TBARs was presented in both gastrocnemius and SOL muscles after just three days of DEX injections versus control group [34]. On the other hand, Jeje and Raji reported higher liver MDA level after chronic 14 and 21 days of DEX treatment when compared with short-term DEX administration [14]. Additionally, it was shown that oxidative stress greatly increased with duration of GC administration, mostly after 3 weeks of treatment [36]. Therefore, in the current study, we expected that CSR would have greater impact on oxidative stress marker level than SSR. We found higher concentration of MDA and 8-iso in both SOL and EDL muscles. Furthermore, our data also showed that marker of protein oxidation, SH groups, was reduced only in the EDL muscle. This observation is consistent with earlier reports documented that white muscles are more susceptible to free radical damage [30]. Moreover, the susceptibility for oxidation of white muscle might be displayed with the result of GSSG level, where the elevation was observed not only in the ST4 but also in the ST2 group. Our data are in line with earlier observations that ROS generation was higher in white muscle versus red muscle [30, 37]. Additionally, it was demonstrated that the EDL muscle shows a greater tendency to atrophy after DEX treatment than the SOL muscle [38]. One of the explanations of this phenomenon can be that the fast-twitch muscles contain higher glucocorticoid receptor (GR) content compared to slow-twitch

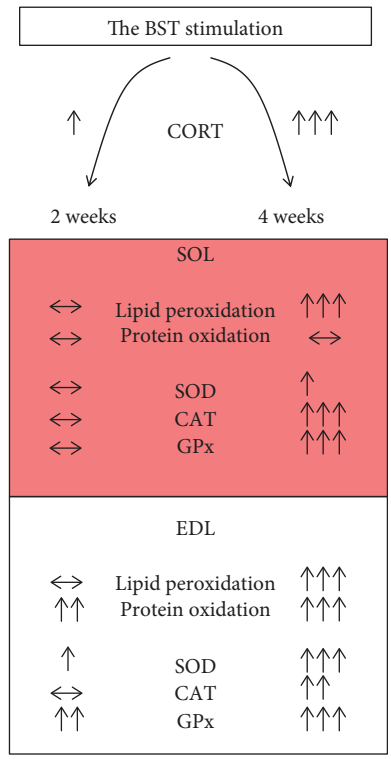

Figure 7: The electrical stimulation of BST activates HPA axis and induces oxidative stress in both types of skeletal muscles during CSR.

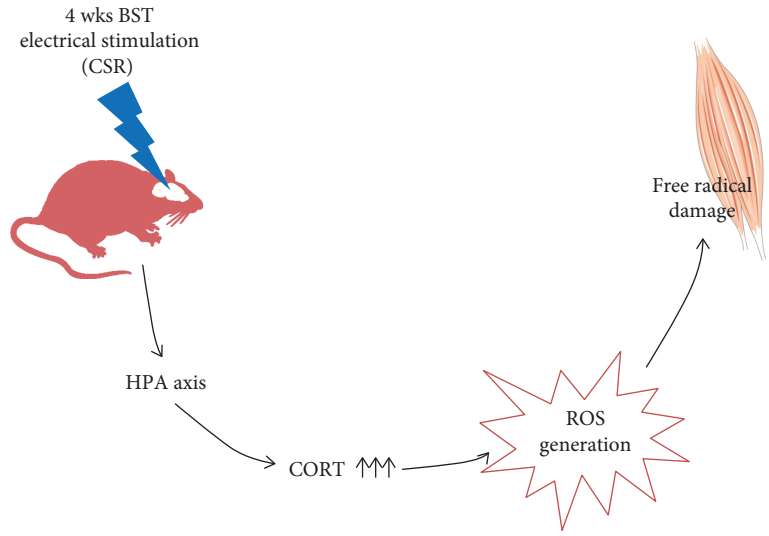

Figure 8: The potential consequences of reaction to the long-term "psychological stress" lead to the free radical damage of skeletal muscle. (1) The bed nucleus of the stria terminalis is linked with the chronic stress response. (2) The chronic BST stimulation induces massive plasma CORT secretion by HPA axis. (3) The chronic stress response evokes ROS generation in two types of skeletal muscles. (4) Lipid and protein peroxidation in muscle is associated with higher CORT level. (5) The activity of antioxidant enzymes is elevated during the chronic stress response.

muscles [39], and therefore it is presumably more susceptible to develop negative effect of GC action. However, opposite conclusion was demonstrated [40], it was suggested that an increased sensitivity of the SOL muscle to GCs occurs only in old rats.

4.2. The Endogenous GC Secretion as a Base of Stress Response. Recently, it was shown that GC secretion might be provoked by different types of stressors [41]. Some of them link oxidative stress in skeletal muscles with hypermetabolism state and with stress response to situations such as burn trauma [42, 43]. However, other pathological conditions, for example, cancer, may stimulate endogenous GC production and contribute to metabolic derangements and the skeletal muscle loss [44, 45]. Likewise, psychological stress is widely associated with ROS generation, mainly in central nervous system, with simultaneous increase of plasma CORT level. Elevated GCs is combined with the development of impairments of cognitive function, learning, and memory [10–13, 33, 46–48]. The evidence for a direct connection was observed between CSR and neurodegenerative disorders such as AD or PD [49–51]. Moreover, one of the earliest events in AD pathogenesis is a systemic oxidative stress, indicated by an increase lipid peroxidation and GSSG in cerebrospinal fluid, plasma, and urine [52]. Taken together, it seems to be worth to consider that both AD and PD show abnormalities not only in structure of central nervous system but also in abnormal functioning of skeletal muscle which cause disorders such as bradykinesia, rigidity, tremor, postural instability, and gait disorders [53, 54]. Therefore, wider perspective to the assessment of oxidative stress in skeletal muscle in neurodegenerative diseases seems to be justified.

4.3. The Antioxidant Enzyme Activity Changes during Stress Response. In the current study, we observed increased activity of main three antioxidant enzymes in the white muscle and two of them (CAT and GPx) in the red muscle. We did not observe any changes in the activity of total and Cu/ZnSOD in the SOL muscle. In contrast with the red muscle, EDL activity of total SOD and both Cu/ZnSOD and MnSOD significantly increased as compared to the SHM and ST2 groups after 4 weeks of stimulation. Further, in the red muscle, GPx activity was higher only in 4-week stimulated group. Interesting observations were made in the white muscle where the activity of GPx was higher not only in the ST4 group but also in the ST2 group compared to the SHM group. This observation corresponds with the level of GSSG and confirms that the EDL muscle is more susceptible to the disruption than the SOL muscle. However, an opposite effect was observed in the studies on the influence of CORT injections on ROS generation in the hippocampus where activity of SOD, CAT, and GPx decreased after CORT treatment compared to a control group [10]. One of the explanations for these differences in antioxidant enzyme activities between the brain and muscle can be that the brain is much more susceptible to GC-induced oxidative stress. Also, the brain is the main target of GCs and generally has low antioxidant capacity, high metabolic activity, and highly susceptible to peroxidation cell membranes [36]. Secondly, it is also well known that differences between red and white muscles occur, and red muscles possess higher antioxidative capacity in terms of higher antioxidant enzyme activity [55], what may provide more effective defense against ROS generation. Another explanation for the increased activity of antioxidant enzymes in our study may be a pathway where ROS induced by GCs activate a signaling cascade, which results in further FOXO activation [9]. It was shown that FOXO may induce gene expression for antioxidant enzymes, that is, MnSOD and CAT but also proteins responsible for protein catabolism [3, 52]. To our knowledge, this is the first

study that shows the influence of BST on regulation of HPA axis activity during CSR with simultaneous oxidative stress in both types of skeletal muscles is a consequence of elevated plasma CORT level (Figure 7).

5. Conclusions

We found elevated level of lipid and protein peroxidation and higher activity of antioxidant enzymes in both SOL and EDL muscles after 4 weeks of stimulation. Moreover, four but not two weeks of electrical stimulation of BST evokes increased plasma CORT level, resulted in oxidative stress in skeletal muscles (Figure 8). Taken into account, higher markers of free radical damage of macromolecules and elevated antioxidant enzyme activity in EDL muscle, we determine that the white muscle is more susceptible for disruption. These data may suggest that the electrical stimulation of the BST could be useful as a novel imitator of the CSR. Therefore, we postulate that the CSR associated with ROS generation may be one of the factors for the development of neurodegenerative diseases in human being. However, further studies are necessary to find the mechanism(s) of macromolecule disruption resulted by the CSR.

Abbreviations

BST: Bed nucleus of the stria terminalis
CAT: Catalase
CORT: Corticosterone
Cu/ZnSOD: Copper/zinc-dependent dismutase
CSR: Chronic stress response
DEX: Dexamethasone
GCs: Glucocorticoids
GPx: Glutathione peroxidase
GR: Glucocorticoid receptor
GSSG: Glutathione disulfide
MDA: Malondialdehyde
MnSOD: Manganese-dependent superoxide dismutase
ROS: Reactive oxygen species
SH: Sulfhydryl groups
SSR: Short stress response.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have no conflict of interest to declare.

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Article

BST Stimulation Induces Atrophy and Changes in Aerobic Energy Metabolism in Rat Skeletal Muscles—The Biphasic Action of Endogenous Glucocorticoids

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Abstract: (1) The primary involvement in stress-induced disturbances in skeletal muscles is assigned to the release of glucocorticoids (GCs). The current study aims to investigate the impact of the biphasic action of the chronic stress response (CSR) induced by the electrical stimulation of the bed nucleus of the stria terminalis (BST) effects on muscle atrophy and aerobic energy metabolism in soleus (SOL) and extensor digitorum longus (EDL) muscles. (2) Male Wistar rats (n = 17) were used. The rats were divided randomly into three groups: the BST two weeks (ST2), four weeks (ST4), and the sham (SHM) electrically stimulated group. The plasma corticosterone (CORT) and irisin concentration were measured. Glucocorticoid and mineralocorticoid receptors (GR and MR), 11β-hydroxysteroid dehydrogenase type 1 and 2 (HSD11B1 and HSD11B2), atrogin-1, and insulin-like growth factor-1 (IGF-1) level were determined in SOL and EDL muscles. Citrate synthase (CS) activity was measured in both muscles. (3) We found elevated plasma concentration of CORT and irisin, raised the level of GR in SOL muscle, and the higher level of MR in both muscles in the ST4 group. The level of HSD11B1 was also higher in the ST4 group compared to the SHM group. Moreover, we observed increased activity of CS in SOL. (4) We suggest that biphasic action of the glucocorticoid induced by the CSR occurs and causes dysregulation of proteins involved in muscle atrophy and aerobic energy metabolism. Our findings potentially contribute to a better understanding of the mechanisms by which GCs and the CSR may regulate muscle atrophy and energy preservation of the red muscle.

Keywords: brain stimulation; glucocorticoids; mineralocorticoid receptor; glucocorticoid receptor; chronic stress; mitochondria

1. Introduction

Stress is one of the most alarming health problems in the modern world. This explains a pressing need for explorations into the biological mechanisms and pathways linking stress and health. It is well documented that the chronic stress response (CSR) leads to extremely negative consequences and is linked to many disease states, affecting the health of many populations. Stress-induced disturbances occur through multiple biochemical and signaling pathways. However, the major involvement in

this process is assigned to the pathways that determine system response to stress through the release of glucocorticoids (GCs) [1,2]. We propose the electric stimulation of the bed nucleus of the stria terminalis (BST) as the model that mimics unconscious stress in rats. Many studies have proved that BST plays a crucial role in the activation of the hypothalamus–pituitary–adrenocortical (HPA) axis during stress [3,4]. Besides, the excess high level of GCs occurs in several pathologic conditions such as diabetes, starvation, cancer, burn injuries, and depression, as well as after long-term medical treatment of synthesized GCs [5].

Under stressful or pathophysiological conditions, circulating GCs levels are greatly increased, which in turn decreases the rate of protein synthesis and rises proteolysis to generate amino acids that serve as precursors for hepatic gluconeogenesis. In skeletal muscles, it leads to the two main adverse effects: firstly, the development of oxidative stress, and secondly, skeletal muscle atrophy and muscle weakness [6]. On the other hand, GCs play a biphasic role on muscle and neuronal mitochondrial dynamic, demonstrating that at a low level, they potentiate; however, at chronic high level, GCs attenuate mitochondrial energy metabolism, respectively [7].

Mounting data indicate that the effects induced by GCs may be caused and regulated independently, at multiple levels of control, by the glucocorticoid receptor (GR) and activity of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) both in vitro and in vivo. The GCs lead their signal mainly through the intracellular GR; however, they may also act through different mechanisms. The major action is to regulate the transcription of its primary target genes through genomic glucocorticoid response elements (GREs) by directly binding to DNA or tethering onto other DNA-binding transcription factors. These GR primary targets trigger physiological and pathological responses of GCs [6]. However, the GC bioavailability and action depend not only upon circulating levels or GR content but also on tissue-specific intracellular metabolism by HSD11B1. Essential metabolic tissues, including liver, adipose tissue, and skeletal muscle express HSD11B1, whose function is to convert inactive cortisone to cortisol or corticosterone (CORT) [8].

The recent study shows the presence and the activity of the mineralocorticoid receptor (MR) in skeletal muscle [9]. MR and GR exhibit cross-reactivity with endogenous GCs, which have the same or even higher affinity to MR than GR (depending on the tissue). As a consequence of the high homology with GR, MR is activated by both mineralocorticoids (aldosterone, deoxycorticosterone) and GCs [9]. Data from the past several years have assigned a specific role for the MR in mediating oxidative stress development. Furthermore, data indicate that ALD/MR-dependent NADPH induces superoxide production with simultaneous increasing serum 8-isoprostane (8-iso) and thiobarbituric acid reactive substances (TBARs) levels [10,11].

The novel report presents that the skeletal muscles may influence adaptation to psychological stress [12], and the central role is assigned to the peroxisome proliferator-activated receptor gamma coactivator 1-alpha–fibronectin type III domain-containing protein 5–brain-derived neurotrophic factor (PGC-1α-FNDC5-BDNF) pathway. Irisin, the PGC-1α-dependent myokine, classically is secreted by skeletal muscle during exercise [13]. However, both GCs and GR might be considered as a positive regulator of FNDC5 expression [14]. Therefore, the question remains whether GCs may cause irisin augmentation as a consequence of the CSR. In particular, irisin flux is higher during high-intensity exercise than during low-intensity exercise, which is accompanied by increased cortisol level in high-intensity efforts [15].

In the present study, we aim to determine whether the CSR induced by the electrical stimulation of the BST effects on muscle atrophy and aerobic energy metabolism in soleus (SOL) (which contains predominantly slow-twitch fibers [16]) and extensor digitorum longus (EDL; containing maximum 10% of type I fibers [16]) muscles of the rat. Therefore, the study investigates the impact of the biphasic action of the CSR on the GR, MR protein content, and the HSD11's B1 and B2 activity as well as mitochondrial function in both muscles. We presume that both augmented receptors and HSD11's B1 and B2 activity are associated with muscle atrophy. We hypothesize that as a result of the biphasic action of GCs during the CSR, muscle atrophy in both muscles will be observed. However, on the

other hand, GCs/insulin-like growth factor 1 (IGF-1) pathway will potentially play protective action in the red muscle manifested by the higher mitochondrial CS activity.

2. Results

2.1. Plasma CORT

Lately, we demonstrated on the same experimental model that rats' plasma CORT level after the BST stimulation significantly increased in the four weeks stimulated group (ST4) group as compared with the two weeks stimulated (ST2) and sham-stimulated (SHM) groups [17]. The results are summarized in Table 1.

Table 1. The corticosterone (CORT) concentration in the plasma.

	SHM (n = 6)	ST2 (n = 6)	ST4 (n = 5)
CORT (ng/mL)	88.6 ± 45.6	170.1 ± 104.1 ***	445.9 ± 31.6 ###

Results were expressed as mean ± SD, *** $p < 0.001$ ST2 vs. ST4, ### $p < 0.001$ SHM vs. ST4.

2.2. GR and MR Content in Skeletal Muscles

Muscle GR content was the highest in the ST4 group in SOL muscle (426.63 ± 77.79 ng/mg protein), and it was significantly lower in the SHM group (280.58 ± 67.39 ng/mg protein; $p < 0.05$). Moreover, the GR concentration was also significantly higher in the ST2 group when compared to the SHM group (424.86 ± 109.46 ng/mg protein; $p < 0.05$). In contrast to the SOL muscle in the EDL muscle, the GR level was the lowest in the ST4 group, and it was 193.88 ± 60.31 ng/mg protein. Also, the highest concentration of GR was observed in the ST2 group (489.49 ± 192.72 ng/mg protein), and the differences were statistically significant vs. both the SHM (282.81 ± 101.32 ng/mg protein) and ST4 groups ($p < 0.05$; Figure 1A).

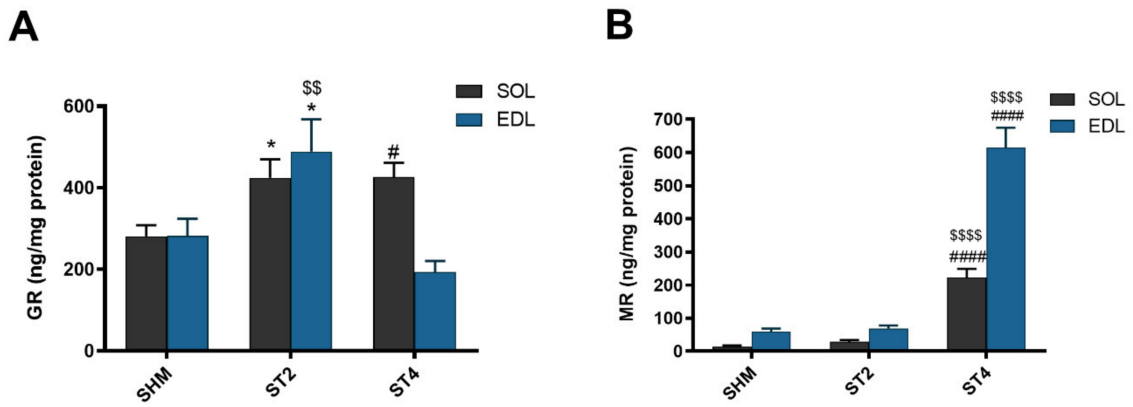

Figure 1. The level of glucocorticoid receptor (GR) (**A**) and mineralocorticoid receptor (MR) (**B**) in soleus (SOL) and extensor digitorum longus (EDL) muscles. Results were expressed as mean ± SEM, sham-stimulated (SHM; n = 6), two weeks stimulated (ST2; n = 6), four weeks stimulated (ST4; n = 5). * $p < 0.05$ SHM vs. ST2, # $p < 0.05$ SHM vs. ST4, $$ $p < 0.01$ ST2 vs. ST4, $$$$ $p < 0.0001$ ST2 vs. ST4, #### $p < 0.0001$ SHM vs. ST4.

The muscular concentration of MR was the highest in the ST4 group, both in SOL and EDL muscles. In SOL, it was 222.48 ± 58.24, 28.98 ± 13.02 and 14.49 ± 8.10 ng/mg protein in the ST4, ST2, and SHM groups, respectively ($p < 0.001$). Also, in the EDL muscle, the concentration of MR was elevated approximately ninefold in the ST4 group (614.34 ± 135.56) as compared to the SHM (59.38 ± 22.80) and ST2 (68.88 ± 22.65 ng/mg protein) groups ($p < 0.001$; Figure 1B).

2.3. HSD11B1 and HSD11B2 Content in Skeletal Muscle

The level of HSD11B1 was the highest in the ST4 group in both muscles. In the SOL muscle, there were 2686.57 ± 629.21 in the ST4, 559.11 ± 161.63 in the ST2, and 533.53 ± 187.49 ng/mg protein in the SHM groups, respectively ($p < 0.001$). A similar observation was made in the EDL muscle, and the values were 1308.83 ± 243.08 in the ST4, 360.58 ± 176.06 in the ST2, and 352.87 ± 104.76 ng/mg protein in the SHM groups, respectively ($p < 0.001$; Figure 2A).

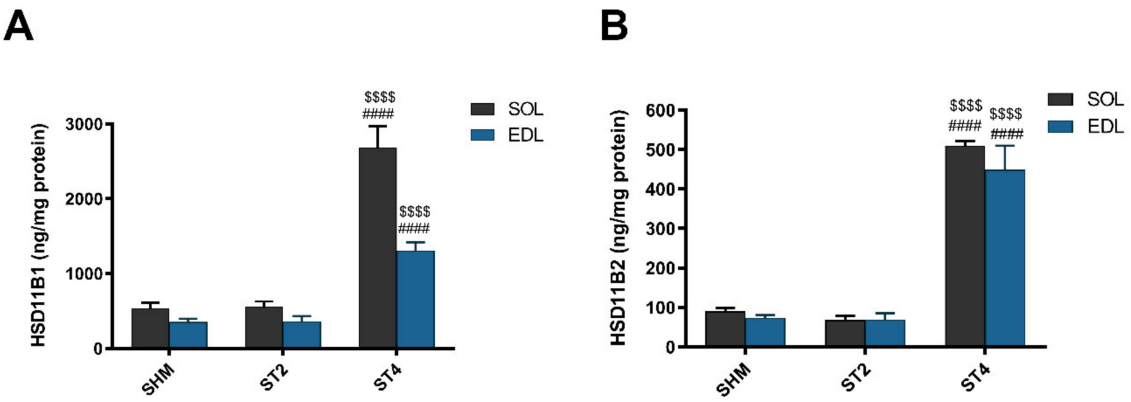

Figure 2. The level hydroxysteroid dehydrogenase type 1 (HSD11B1) (**A**) and hydroxysteroid dehydrogenase type 2 (HSD11B2) (**B**) in SOL and EDL muscles. Results were expressed as mean ± SEM, SHM ($n = 6$), ST2 ($n = 6$), ST4 ($n = 5$). $^{\$\$\$\$}$ $p < 0.0001$ ST2 vs. ST4, $^{\#\#\#\#}$ $p < 0.0001$ SHM vs. ST4.

Also, the muscle HSD11B2 level was the highest in the ST4 group when compared to the SHM and ST2 groups in both muscles. The differences between groups were statistically significant, and in details in the SOL, it was 509.73 ± 25.91, 67.82 ± 26.50, and 90.48 ± 19.81 ng/mg protein in the ST4, ST2, and SHM groups, respectively ($p < 0.001$). In EDL the highest level of HSD11B2 in the ST4 group was observed (448.85 ± 135.96 ng/mg protein), and the differences were statistically significant when compared the ST2 and SHM groups (ST4 vs. SHM $p < 0.001$, and ST4 vs. ST2 $p < 0.001$; Figure 2B).

2.4. Marker of Muscle Atrophy and IGF-1 Content

After four weeks of rat brain stimulation, the marker of muscle atrophy was elevated in the ST4 group when compared to the ST2 and SHM groups in both muscles. Atrogin-1 (FBXO32) was higher in SOL muscle, and the values were 100.86 ± 20.12 in the ST4, 56.52 ± 16.95 in the ST2, and 62.20 ± 13.49 pg/mg protein in the SHM groups, respectively ($p < 0.01$). In the EDL muscle, significant differences were observed between the ST4 and the SHM groups ($p < 0.01$; Figure 3A) and between the ST4 and the ST2 groups ($p < 0.001$). The average level of atrogin-1 was 67.77 ± 17.60, 32.70 ± 6.42, and 40.61 ± 5.40 pg/mg of protein in the ST4, ST2, and SHM groups, respectively.

In SOL, IGF-1 concentration was the highest in the ST4 group, 220.07 ± 29.15, and it was significantly lower in the ST2 (114.74 ± 19.19 ng/mg protein) and the SHM (124.83 ± 19.64 ng/mg protein) groups ($p < 0.001$; Figure 3B). We did not observe any statistically significant differences between the groups in the EDL muscle, and the values were 140.49 ± 38.99 in the ST4, 117.63 ± 55.69 in the ST2, and 113.55 ± 24.23 ng/mg protein in the SHM groups, respectively.

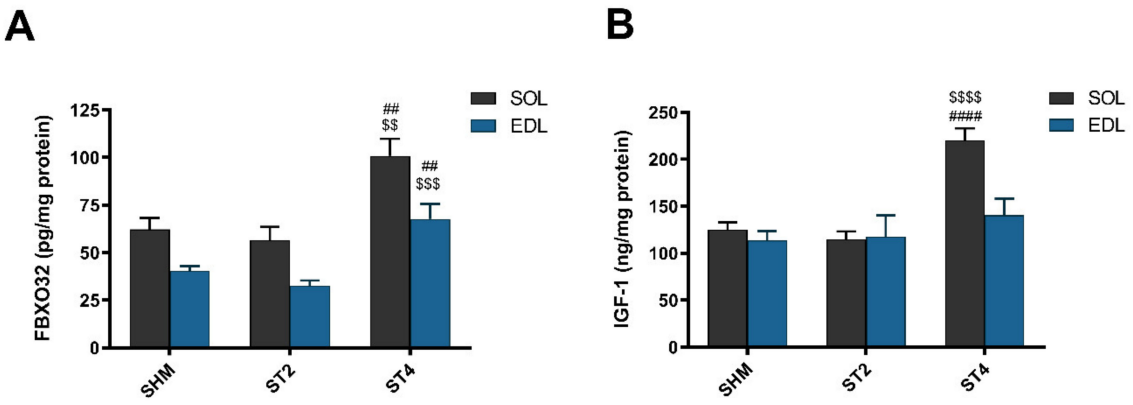

Figure 3. The level atrogin-1 (FBXO32) (**A**) and insulin-like growth factor-1 (IGF-1) (**B**) in SOL and EDL muscles. Results were expressed as mean ± SEM, SHM (n = 6), ST2 (n = 6), ST4 (n = 5). $^{\#\#}$ $p < 0.01$ SHM vs. ST4, $^{\$\$}$ $p < 0.01$ ST2 vs. ST4, $^{\$\$\$}$ $p < 0.001$ ST2 vs. ST4, $^{\$\$\$\$}$ $p < 0.0001$ ST2 vs. ST4, $^{\#\#\#\#}$ $p < 0.0001$ SHM vs. ST4.

2.5. CS activity in Skeletal Muscle and Irisin Concentration in Plasma

The activity of CS was not different in the EDL muscle after brain stimulation. Nevertheless, we observed statistically significant differences in the SOL muscle between the ST4 (65.30 ± 2.14) and the SHM (61.54 ± 3.30 nmol/min/mg of protein) groups ($p < 0.05$; Figure 4).

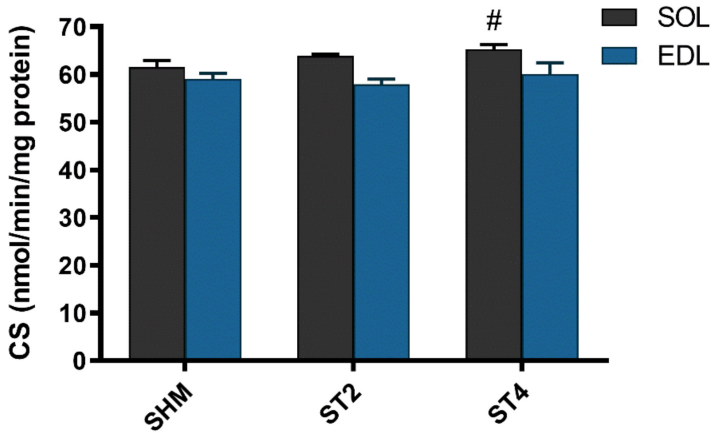

Figure 4. The citrate synthase (CS) activity in SOL and EDL muscles. Results were expressed as mean ± SEM, SHM (n = 6), ST2 (n = 6), ST4 (n = 5). $^{\#}$ $p < 0.05$ SHM vs. ST4.

The irisin level significantly differed between the groups, and the highest concentration was observed in the ST4 (131.58 ± 11.86 ng/mL) as compared to the ST2 and SHM (109.96 ± 8.57 and 91.41 ± 10.30 ng/mL, respectively). Moreover, the irisin level was also significantly higher in the ST2 group than in the SHM group ($p < 0.05$, Figure 5).

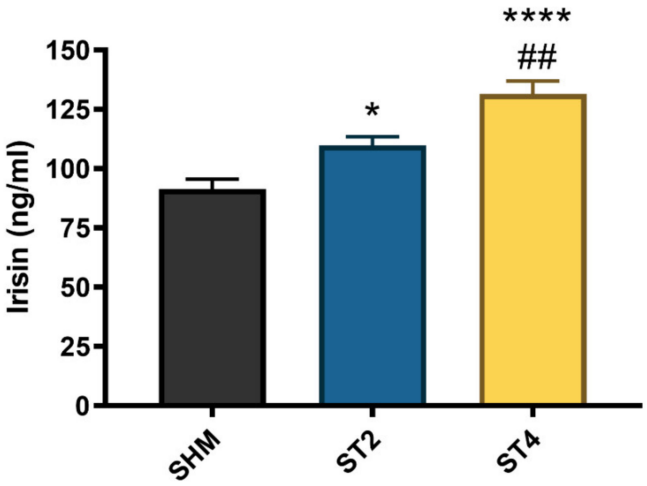

Figure 5. The plasma irisin level. Results were expressed as mean ± SEM, SHM (n = 6), ST2 (n = 6), ST4 (n = 5). * $p < 0.05$ SHM vs. ST2, ## $p < 0.01$ ST2 vs. ST4, **** $p < 0.0001$ SHM vs. ST4.

3. Discussion

We demonstrate that the biphasic action of CORT and the CSR caused the skeletal muscle atrophy measured by atrogin-1 level in both types of skeletal muscles with the simultaneously elevated level of HSD11B1, HSD11B2, and MR. In addition, we noticed a higher level of GR after 4 weeks in the CSR group as compared to the SHM group but solely in the red muscle. On the other hand, we found a higher IGF-1 level and an elevated CS activity only in SOL muscle. Additionally, the highest concentration of irisin in blood in the ST4 group was observed. Our findings have the potential to contribute to a complete understanding of mechanisms by which CORT and the CSR may regulate muscle atrophy, as well as preservation in the red, and devastation in the white muscles.

According to our knowledge, this is the first preclinical work that shows the interplay between HSD11B (type 1 and 2) and GR/MR in the CSR conditions. Obtained data show that the CSR linked with the elevated CORT level [17] inducing the skeletal muscle atrophy is associated with the FOXO/atrogin-1 pathway. Our observations confirm the previously published data, which clearly shows that HSD11B1 may be a major regulator of the muscular atrophy. For instance, increased HSD11B1 activity in skeletal muscle is linked with the development of insulin resistance, a decrease of muscle mass, and elevated gene expression associated with muscle atrophy [18]. However, the opposite effect was observed in the experiment conducted on HSD11B1 knockout mice. The level of skeletal muscle atrophy markers (MuRF-1 and atrogin-1) was lower in HSD11B1 KO mice as compared to the control group after CORT administration [8].

Furthermore, Zhao and co-workers proved that reduced GR expression in C2C12 myotubes compromises GC-reduced protein degradation [19]. Also, it is known that fast-twitch muscles contain higher GR content as compared to slow-twitch muscles [20]. It implies a conviction that a white muscle is more susceptible to develop adverse effects of GCs action. Our observation of a higher level of protein oxidation in EDL, as compared to SOL muscle, in the CSR rats partially proves previous results [17]. On the other hand, our results show that in EDL, not GR but MR content is dominant, and could be a significant contributing factor in muscle devastation. Some authors have postulated that the inhibition of MR improved skeletal muscle function and pathology in DMD mice [9] and prevented sarcopenia in older people [21]. Moreover, data reported that GR–MR interactions do occur and that they have functional consequences on gene transcription, including increasing gene upregulation and downregulation. In this way, the interactions between GR and MR can enhance the magnitude of the transcriptional response to GCs [22], and this can be a partial explanation of the biphasic effects of GCs on EDL and SOL muscles.

Apart from protein degradation in skeletal muscle cells, it should be noted that many GC-induced products also can repress protein synthesis through various mechanisms. It is believed that by mainly blocking the transport of amino acids into the muscle, this inhibits the stimulatory action of insulin-like growth factor 1 (IGF-1), and the influence of amino acids on mammalian target of rapamycin (mTOR) through the induction of regulated in development and DNA damage responses 1 (REDD1) and Kruppel-like factor 15 (KLF15) [23]. Surprisingly, we did not observe an expected decrease in IGF-1 level, but we have found an elevation of the concentration of that particular protein, but only in red muscle. However, some reports show that under certain conditions, like immobilization, muscle denervation [24], or in atrophy models induced by proinflammatory cytokines [25], IGF-1 does not prevent muscle cell atrophy, and its level may stay unaltered.

Lately, it was reported that ROS generation is involved in the aging process and dysfunction in several metabolic and neurodegenerative diseases, which may be partially connected with elevated GCs level [26]. In our recent work, it was presented that the CSR induced by the electrical stimulation of the BST causes the elevated level of markers lipid and protein peroxidation in EDL muscle. However, no changes in protein oxidation but only the higher level of lipid peroxidation marker in the SOL muscle was found [17]. Furthermore, the oxidation of sulfhydryl groups likely contributes to the deactivation and degradation of mitochondrial enzymes and transport proteins [27]. Possibly, that elevated protein damage observed in white, not in red muscle, might partially explain the biphasic action of the excessive flux of endogenous GCs. However, most of the available data related to skeletal muscles have been based on models with synthetic GCs administration, rather than the induction of endogenous GCs secretion. It seems to be worth noting because there is evidence indicating that administering exogenous GCs to mimic a condition of physiological stress may not reflect a realistic condition whether circulating GCs may attain well above peak levels observed during a stress response [28].

In the present study, we found the increased activity of CS in red muscle, but not in white muscle in the CSR conditions. These findings are consistent with previous observations from animals and humans experiments. Thus, in detail, Koerts-de Lang and co-workers show that CS activity in the tibialis anterior was significantly higher in the rats treated with triamcinolone compared to the control and prednisolone-treated rats [29]. Similar effects (statistically insignificant) were observed in tibialis anterior from patients with severe COPD treated with prednisolone [30]. What is more, Weber and co-workers showed a 2.5-fold induction of cytochrome c oxidase activity in myotubes treated with dexamethasone (DEX). They presented 2-fold elevated levels of mitochondrial mRNAs and a 2-fold increase of cytochrome c oxidase activity in quadriceps muscle of rats after treatment with DEX [31]. However, several studies show a lack of abnormalities or even a decreased activity of CS [32,33] but in the deltoid and quadriceps muscles. These discrepancies may be associated with different composition of muscle fibers or with the concentration of endogenous or synthetic GCs in the mentioned studies or unknown factors. However, the current study demonstrates the partially protective effect of CORT on SOL muscle accompanying elevated CS activity in the ST4 group.

Additionally, the activity of CS in EDL did not differ between the groups, which are also consistent with the data mentioned above. It was reported that high levels of circulating GCs stimulate mitochondrial biogenesis, and that among the cell types chosen for analysis, this occurrence is rather specific for skeletal muscle [31]. Hence, the question arises of why this phenomenon only affects red muscle? We observed the elevated GR level in the stressed rats only in SOL muscle. A possible mechanism could be linked with the fact that only GR (as opposed to MR) is found in mitochondria, suggesting that GRs drive the direct actions of glucocorticoids on mitochondria [34].

Furthermore, higher CS activity in SOL muscle is necessary because as energy-demanding by mitochondria to cover the expanded ATP-turnover in this stressful condition. This higher CS activity in red muscle is associated with higher irisin flux in serum and likely with augmented PGC-1α content in skeletal muscle. Recently, in work from our laboratory, we demonstrated that a relationship between the PGC-1α and CS activity exists as an indirect marker of mitochondrial biogenesis [35]. Additionally,

we observed the significantly higher level of IGF-1 but only in SOL muscle, and some researchers postulate that IGF/PI3K/AKT signaling also plays a crucial role in mitochondrial biogenesis [36].

Besides, we can also suggest that the increased activity of CS in the SOL muscle might be linked with the elevated irisin level in plasma in the ST4 and ST2 groups. Research shows that both cortisol and GR might be considered as a positive regulator of FNDC5 expression [14], and that stays in line with our results (the highest level of CORT and GR in the ST4 group). Moreover, numerous studies indicate the presence of the PGC1-α/FNDC5/Irisin pathway [37,38], and that elevation in the FNDC5 gene expression in skeletal muscles is dependent on PGC-1α [13]. The PGC-1α, in turn, is thought about a mitochondrial biogenesis modulator [39], and this can be used to explain the possible indirect mechanism of higher CS activity in red muscle in the CSR conditions.

4. Materials and Methods

4.1. Animals

The animals used in the experiment were previously described by Karnia and co-workers [17]. Briefly, male Wistar rats (n = 17) weighing 250–300 g were used. They were provided with a standard diet ad libitum with free access to tap water and were maintained on a 12 h light/dark cycle, temperature 22 °C, humidity 50–55%.

4.2. Study Design

The study design was previously described by Karnia and co-workers [17]. In brief, the habituation of the animals was conducted daily for about two weeks before the experiment to minimize stress caused by the experimental procedures. All procedures were performed between 8:00 and 12:00. The rats were divided randomly into three groups: the BST two weeks electrically stimulated group (ST2; n = 6), the BST four weeks electrically stimulated group (ST4; n = 5), and the BST sham group (SHM; operated but not stimulated, n = 6).

The experiments were carried out according to the European Communities Council Directive (86/609/EEC), and the protocols were approved by the Local Animal Research Ethical Committee for the Care and Use of Laboratory Animals at the Medical University in Gdansk, Poland (No 8/2010).

4.3. Surgery and Electrical Stimulation

Standard stereotaxic surgery and the electrical stimulation were performed following the procedure described before [40]. Briefly, two weeks after recovery from the surgery, the stimulated groups were screened for BST stimulation-induced behavior. Once determined, the current intensity was held constant throughout 14 (ST2) or 28 (ST4) consecutive days of the stimulation. The animals from the SHM group were treated in the same way as the experimental group, with the exception that no current was passed through the electrodes.

4.4. Animal Sacrifice, Blood and Muscle Collection

All animals were sacrificed at a required time point (one hr after the termination of the last electric stimulation). The blood samples were collected by heart puncture one hour after the last session of stimulation. The blood was centrifuged at 2000× g for 10 min at 4 °C. Plasma was separated and stored at −80 °C for later analysis. EDL and SOL muscles were removed from both hind limbs following the procedure described before [17].

4.5. Muscle Homogenization

Prior to the chemical assays, muscles were minced and homogenized in an ice-cold buffer that contained 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM DTT, 1.15% KCl and 1:200 protease inhibitor (Sigma Aldrich P8340) at pH 7.4. The homogenates were then centrifuged at 750× g at 4 °C for 10 min. Then the supernatant was divided into serial aliquots for enzyme activity and enzyme-linked

immunosorbent assay (ELISA) measurements. Samples for ELISA were additionally centrifuged at 5000× *g* for 10 min. Protein concentration was determined using the Bradford assay (Sigma, B6916) according to the manufacturer's instructions.

4.6. ELISA Assays

The following ELISA kits were used in the experiment and were determined by the supplied manufacturer's instruction:

Irisin (EK-067-16, Phoenix Pharmaceuticals, Inc., USA) was measured in plasma.

11β-Hydroxysteroid dehydrogenase type 1 (HSD11B1; E2268r), 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2; E11618r), glucocorticoid receptor (GR; E1608r), mineralocorticoid receptor (MR; E0158r, EIAab, China), atrogin-1 (FBXO32; ER1518, Fine test, China) and insulin-like growth factor 1 (IGF-1; SEA050Ra, Cloud-Clone, USA) were performed in 5000× *g* supernatant of both muscles.

4.7. Citrate Synthase (CS) Activity

CS activity was measured in accordance to work by Dzik and co-workers [35]. In brief, 50 µL of the supernatant of SOL or EDL (4%; 750× *g*) was added to 790 µL of buffer (0.1 M Tris-HCl, 5 mM EDTA, 0.05% Triton X100, pH 8.1), plus 100 µl of freshly made DTNB (1 mM), 10 µL acetyl-CoA (10 µM), and 50 µL of freshly made oxaloacetic acid (10 mM) to initiate the reaction. The reactions were performed at 37 °C in duplicate, and absorbance was read at 412 nm in a spectrophotometer (CE9200, Cecil Instruments Limited, Cambridge, UK). CS activity was expressed as nmol/min/mg of protein.

4.8. Statistical Analysis

Statistical analyses were performed using a software package (Statistica v. 13.1, StatSoft Inc., Tulsa, OK, USA). One way analysis of variance (ANOVA) was performed, and a Tukey post-hoc test was used for multiple comparisons. The results are expressed as mean ± SD, p values less than 0.05 were considered statistically significant.

5. Conclusions

In conclusion, this study demonstrates for the first time that CORT and the CSR exert major, biphasic effects on muscle atrophy and preservation of muscle devastation in red muscle. Also, to regulate aerobic energy metabolism, there may well be other mechanisms operative in the biphasic effects that GCs exerted on the muscle function. For instance, the dependent concentration–time impact of CORT on MR and GR may explain the biphasic effects of GCs on the pathophysiological role in the different types of skeletal muscle. Moreover, consistent with the findings presented here, previous work showed that elevated CORT induced lipid and protein peroxidation by ROS generation, especially in EDL muscle. On the other hand, it is possible that through GCs/IGF-1 pathway, GR mediates protective effects via different mechanisms in SOL muscle. While the different effects of CORT on MR and GR are undoubtedly crucial for various functions, the regulation of mitochondria function by GCs provides new insights to explain the biphasic effects of GCs in muscles function. Likewise, we propose the potential mechanisms of how GCs and the CSR regulate critical metabolic pathways associated with muscle atrophy (Figure 6A) and preservation of SOL muscle function (Figure 6B) as energy-demanding by mitochondria to cover the expanded ATP-turnover. The possibility that the GCs/IGF-1 pathway, which enhances the function of mitochondria, may be useful in countering the deleterious effects of excessive GCs flux observed in the CSR is an exciting prospect for future investigation. Furthermore, this also raises the intriguing problem-related in the regulation and inhibition of GR and MR action or HSD11B1 activity, as adjunctive therapy to limit the adverse effects associated with GCs excess.

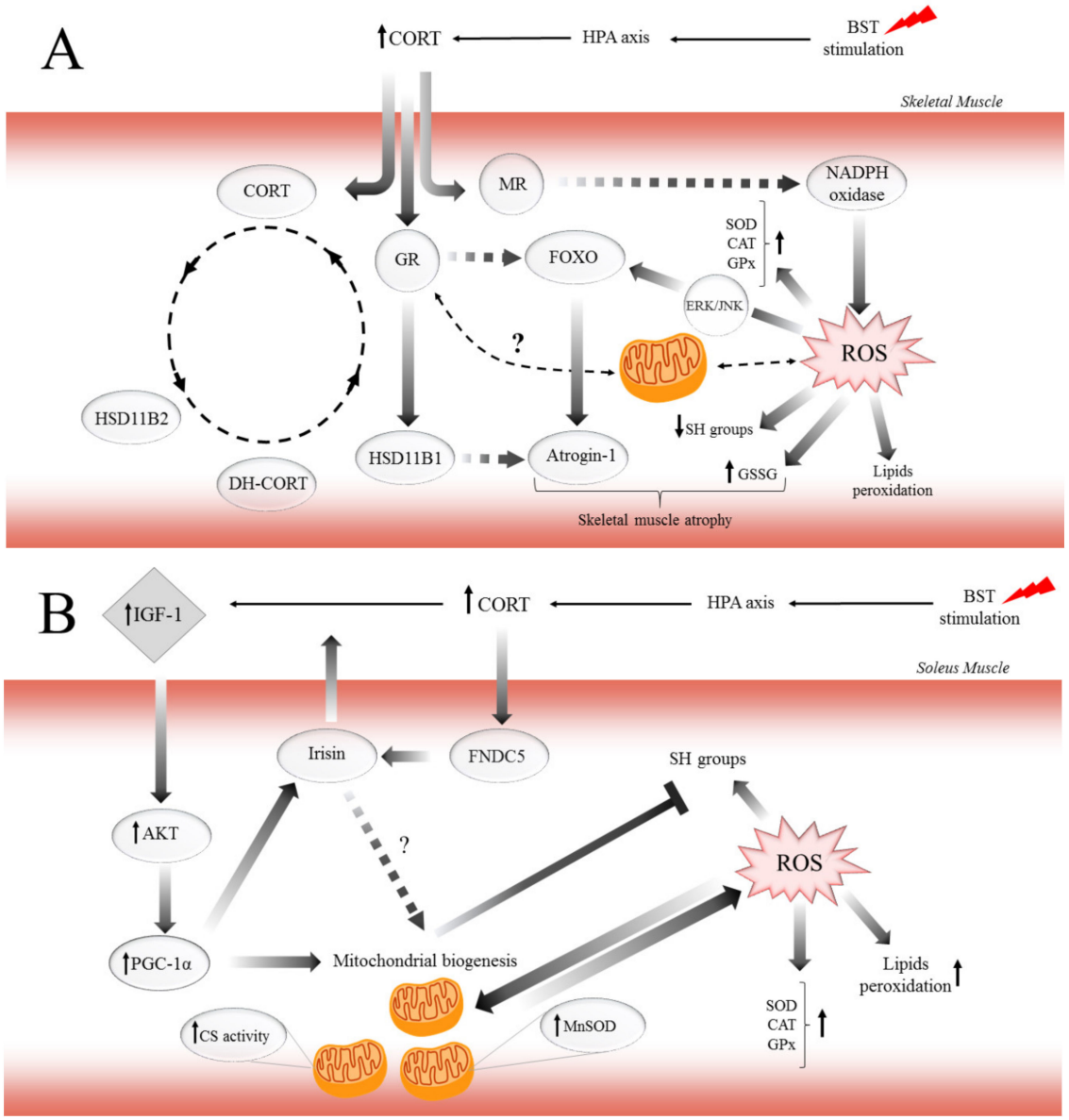

Figure 6. The potential mechanism of biphasic action of CORT-related changes in both (**A**) and only SOL (**B**) muscles during the CSR. (**A**) The BST stimulation mimics the CSR condition and activates the hypothalamus–pituitary–adrenocortical (HPA) axis, which results in CORT secretion. Increased circulating CORT level triggers signaling cascades, including the action of GR (thereby arising in HSD11B1 and fibronectin type III domain-containing protein 5 (FNDC5)/irisin expression), and MR (which might activate NADPH oxidase, causing the ROS generation). Furthermore, GR induces skeletal muscle atrophy in a direct (through FOXOs), and indirect (through HSD11B1-dependent atrogin-1/MURF) mechanism. Additionally, the oxidative damage of macromolecules (decreased level of SH groups, increased lipids peroxidation) occurs with simultaneous activation of enzymatic defense mechanisms (increased activity of SOD, CAT, and GPx). (**B**) In contrast to the adverse changes triggered by the CSR, the level of IGF-1 increased in red muscle and possibly induced the mitochondrial biogenesis through the IGF/PI3K/AKT pathway. Additionally, we observed increased irisin flux, and that protein traditionally is supposed to be a PGC-1α-dependent myokine; however, GR is also a positive regulator of FNDC5 gene expression. We suggest that irisin acts not only in an endocrine but also in an autocrine manner. Hence, irisin also promotes mitochondrial biogenesis in (red) skeletal muscle (manifested, i.e., by increased CS activity) and could be a part of the protective mechanism in red muscle.

Study Limitation

In the current study, the FOXOs and PGC-1α content would give a broader and more precise picture of the described mechanisms. However, we were limited by the amount of biological material, and we had to make the most reasonable choice of protein to be determined. According to work from our laboratory [35], the atrogin-1 level was strongly related to FOXO content in humans' skeletal muscles. Also, the relationship between the PGC-1α and CS activity as an indirect marker of mitochondrial biogenesis was observed. Because of this, we believe that a partly simplified explanation in our recent work is acceptable. Nevertheless, further investigations are necessary.

Author Contributions: Conceptualization, M.J.K. and J.J.K.; Data curation, M.J.K. and D.M.; Formal analysis, M.J.K. and J.J.K.; Funding acquisition, M.J.K. and J.J.K.; Investigation, M.J.K., D.M., K.P.D., D.J.F., and M.P.; Methodology, M.J.K., D.M., and K.P.D.; Project administration, M.J.K.; Resources, J.J.K.; Supervision, J.J.K.; Visualization, M.J.K.; Writing—original draft, M.J.K. and J.J.K.; Writing—review and editing, M.J.K. and J.J.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BST	Bed nucleus of the stria terminalis
CAT	Catalase
CORT	Corticosterone
Cu/ZnSOD	Copper/zinc-dependent superoxide dismutase
CS	Citrate synthase
CSR	Chronic stress response
DEX	Dexamethasone
DH-CORT	11-dehydrocorticosterone
GCs	Glucocorticoids
GPx	Glutathione peroxidase
GR	Glucocorticoid receptor
GSSG	Glutathione disulfide
HSD11B1	11β-hydroxysteroid dehydrogenase type 1
HSD11B2	11β-hydroxysteroid dehydrogenase type 2
IGF-1	insulin-like growth factor 1
MDA	Malondialdehyde
MR	Mineralocorticoid receptor
MnSOD	Manganese-dependent superoxide dismutase
ROS	Reactive oxygen species
SH	Sulfhydryl groups

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The Positive Impact of Vitamin D on Glucocorticoid-Dependent Skeletal Muscle Atrophy

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Abstract: (1) The study aimed to investigate whether vitamin D_3 supplementation would positively affect rats with glucocorticoids-induced muscle atrophy as measured by skeletal muscle mass in two experimental conditions: chronic dexamethasone (DEX) administration and a model of the chronic stress response. (2) The study lasted 28 consecutive days and was performed on 45 male Wistar rats randomly divided into six groups. These included two groups treated by abdominal injection of DEX at a dose of 2 mg/kg/day supplemented with vegetable oil (DEX PL; $n = 7$) or with vitamin D_3 600 IU/kg/day (DEX SUP; $n = 8$), respectively, and a control group treated with an abdominal injection of saline (CON; $n = 6$). In addition, there were two groups of rats chronically stressed by cold water immersion (1 hour/day in a glass box with 1-cm-deep ice/water mixture; temperature ~4 °C), which were supplemented with vegetable oil as a placebo (STR PL; $n = 9$) or vitamin D_3 at 600 IU/kg/day (STR SUP; $n = 9$). The last group was of sham-stressed rats (SHM; $n = 6$). Blood, soleus, extensor digitorum longus, gastrocnemius, tibialis anterior, and quadriceps femoris muscles were collected and weighed. The heart, liver, spleen, and thymus were removed and weighed immediately after sacrifice. The plasma corticosterone (CORT) and vitamin D_3 metabolites were measured. (3) We found elevated CORT levels in both cold water-immersed groups; however, they did not alter body and muscle weight. Body weight and muscle loss occurred in groups with exogenously administered DEX, with the exception of the soleus muscle in rats supplemented with vitamin D_3. Decreased serum 25(OH)D_3 concentrations in DEX-treated rats were observed, and the cold water immersion did not affect vitamin D_3 levels. (4) Our results indicate that DEX-induced muscle loss was abolished in rats supplemented with vitamin D_3, especially in the soleus muscle.

Keywords: dexamethasone; chronic stress; corticosterone; cold water immersion; soleus

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1. Introduction

Skeletal muscle accounts for approximately 40% of body mass and is a major target organ for glucocorticoids (GCs). Under stressful or pathophysiological conditions such as starvation, cancer, or coldness, circulating GC levels are greatly increased. Likewise, the long-term or high-dose administration of synthetic GCs such as dexamethasone (DEX) may lead to decreased protein synthesis and increase proteolysis to generate amino acids to serve as precursors for hepatic gluconeogenesis. In the skeletal muscles, this leads to many adverse effects, particularly skeletal muscle atrophy and muscle weakness [1].

Moreover, GC activity may differ depending on whether it is administered externally or is of internal origin in relation to the stress response [2]. As data show, administering

exogenous GCs like DEX to mimic a condition of physiological stress may not reflect a realistic condition to determine whether circulating GCs may attain the well-above peak levels observed during a stress response [3].

Vitamin D is considered to be a potent anti-catabolic compound [4,5]. Numerous studies suggest a positive role of vitamin D in sarcopenia prevention [6] or inhibition of muscle atrophy by suppression of forkhead box protein O1 (FOXO-1) transcriptional activity [7]. Some studies also imply that vitamin D has antioxidant potential both in the central nervous system [8] and skeletal muscles [9]. In work from our laboratory, we demonstrated that vitamin D deficiency induced protein peroxidation and atrophy in paraspinal muscle, and supplementation with vitamin D reversed those negative alterations [10,11]. On the other hand, the beneficial effect of vitamin D supplementation on skeletal muscle mass is questioned, and some research has indicated that vitamin D supplementation has little or no effect on muscle mass [12,13].

To our knowledge, there are no direct data on the effect of vitamin D_3 on GC-induced muscle atrophy; however, there is some research focused on GC-induced osteoporosis [14] and the influence of the vitamin D analogs in the context of GC-dependent myopathy [15,16]. The exact effect that GCs have on vitamin D_3 metabolism remains ambiguous. For instance, one study indicates that $1,25(OH)_2D_3$ (calcitriol) stimulates (in human adipocytes) the expression of 11β-hydroxysteroid dehydrogenase type 1 (HSD11B1) [17]. The same research shows that calcitriol may act through a rapid, non-genomic mechanism that also stimulates GC release in adipocytes by increasing Ca^{2+} through 1,25-D3-membrane-associated rapid-response steroid binding (1,25-D3-MARRS) and, in consequence, increases the availability of GCs. Moreover, there is a report showing that calcitriol increases oxidative stress in cultured murine and human adipocytes [18]. Nevertheless, another study has shown a positive and highly selective type of activity of vitamin D, inducing oxidative stress only in malignant cells while sparing healthy cells [19].

In addition, serum vitamin D_3 deficiency attenuated the protein content of vitamin D receptor (VDR), with a simultaneous elevated level of peroxidation markers of lipids and proteins in multifidus muscle [11]. There is also evidence linking vitamin D_3 deficiency with GC administration. Data showed that patients who reported GC treatment were twice as likely to have vitamin D deficiency as compared to those without steroid use [20].

Furthermore, calcitriol is considered a true steroid hormone, and like GCs, it may exert several activities in many tissues and organs, demonstrating a synergistic effect in combined therapy [21]. An excellent example of this pharmacological approach is the use of calcitriol and DEX in patients with rheumatoid arthritis, where synovial fibroblast activation is abolished; this combination suppresses the expression of proinflammatory cytokines [22].

Vitamin D_3 has been reported to suppress FOXO-1 transcriptional activity [7], and deficiency of vitamin D_3 might induce skeletal muscle atrophy [10]. However, it is not clear whether vitamin D_3 could prevent GC-induced muscle loss in vivo. Thus, the current study aimed to explore whether vitamin D supplementation attenuated detrimental changes as measured by the body and skeletal muscle weight in chronic DEX-administered rats. We also suspected that cold water immersion as a model of the chronic stress response (CSR) would induce an exogenous GC surge and, in consequence, cause similar deleterious effects. Therefore, supplementation with vitamin D would reverse the adverse effect induced by elevated GCs in the CSR.

2. Materials and Methods

2.1. Animals

The study was performed on 45 male Wistar rats (weighing approximately 300–400 g) obtained from the Medical University of Gdansk, Poland. For the whole experiment the animals were housed 3–4 per cage with food and water provided ad libitum, with a 12-h light/dark cycle and controlled environmental conditions: temperature 22 °C and

humidity 55%. Studies were conducted with the consent of the Local Bioethics Committee in Bydgoszcz, Poland (No. 12/2019), according to European guidelines.

2.2. Study Design

Two weeks before the experiment, animals were handled to acclimate and minimalize stress. Next, rats were randomly divided into 6 groups. Two of these groups were treated with an abdominal injection of dexamethasone (Dexamethasone D4902, Sigma–Aldrich, MN, USA) at 2 mg/kg/day supplemented with vegetable oil (DEX PL; n = 7), or vitamin D_3 (DEX SUP; n = 8). The control group was treated using an abdominal injection of saline (CON; n = 6). Two groups of rats chronically stressed by cold water immersion were given supplementation with vegetable oil as a placebo (STR PL; n = 9) or vitamin D_3 (STR SUP; n = 9). The last group comprised sham-stressed (warm water-immersed) rats (SHM; n = 6).

The STR PL and STR SUP groups were exposed over 28 days to chronic stress by isolation in the glass box (21 × 15 × 30 cm) for 1 hour per day with a 1-cm-deep ice/water mixture (0–4 °C), and the SHM group was placed in sham stress conditions (warm water (35 °C)). The animals from the STR PL and DEX PL groups received oral administration of the vegetable oil as a placebo, and the STR SUP and DEX SUP groups were supplemented with vitamin D_3 at 600 IU/kg (Juvit D3, PPF HASCO-LEK. SA., Poland) for 28 consecutive days.

2.3. Blood Collection

Blood was collected at 2-time points, prior to and after 28 days of the experiment. Blood was taken from the tail vein during isoflurane anesthesia. Samples were centrifuged at 2000× g for 10 min at 4 °C. Serum samples were separated and stored at −80 °C until later analysis.

2.4. Tissue Collection

Soleus, extensor digitorum longus, gastrocnemius, tibialis anterior, and quadriceps femoris muscles were collected from both hind limbs, weighed, and snap-frozen in liquid nitrogen and kept at −80 °C for later analysis. The heart, liver, spleen, and thymus were excised and weighed immediately after sacrifice.

2.5. Biochemical Analysis

2.5.1. Corticosterone Level

According to the manufacturer's instructions, the plasma corticosterone level was determined with a Corticosterone rat/mouse ELISA Kit (DEV9922, Demeditec Diagnostics GmBH, Kiel, Germany). The concentration of CORT was expressed in nanograms per milliliter of plasma.

2.5.2. Vitamin D-25(OH)D_3, 3-epi-25(OH)D_3, 25(OH)D_2, 24.25$(OH)_2D_3$ Levels

Analysis of the vitamin D_3 metabolite levels was performed using the isotope dilution method by the liquid chromatography coupled with tandem mass spectrometry technique (LC-MS/MS). Samples were prepared and analyzed using the Eksigent ExionLC analytical HPLC system with a CTC PAL autosampler (Zwinger, Switzerland) coupled with QTRAP® 4500 MS/MS system (Sciex, Framingham, MA, USA) according to the procedure described previously [23].

2.6. Statistical Analysis

All statistical analyses were performed using the GraphPad Prism 8.3 software program (GraphPad Software, CA, USA). The results are expressed as mean ± SD. The differences between groups were tested using one-way ANOVA followed by the Tukey post-hoc test; p-values less than 0.05 were considered statistically significant.

3. Results

3.1. Plasma Corticosterone (CORT) Level

As was expected, cold water immersion treatment caused a significant induction in hypothalamic–pituitary–adrenal (HPA) axis activation and a CORT surge into the blood flow. Plasma CORT level significantly increased in both (placebo and supplemented) stressed groups of rats. The levels were 403.54 ± 49.73 in the stressed placebo (STR PL) and 359.67 ± 46.32 ng/mL in the stressed supplemented with vitamin D_3 (STR SUP) groups, respectively. There were no differences from the baseline in the control sham-stressed (SHM) rats. In order to assess the correctness of the selection of sham stress conditions, we also determined the CORT level in the control (CON) group, and no changes in that group were observed (Figure 1).

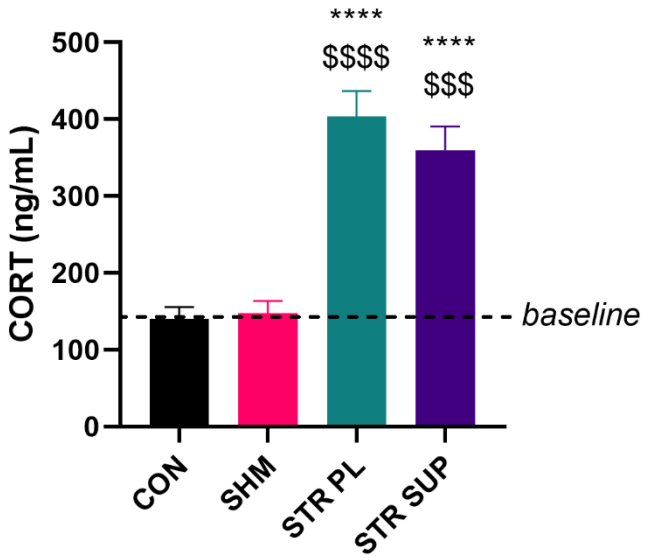

Figure 1. The level of corticosterone (CORT) in plasma. Results are expressed as mean ± SEM. CON (n = 6), SHM (n = 6), STR PL (=9), STR SUP (n = 9), **** $p < 0.0001$ vs. CON, $$$$ $p < 0.0001$ vs. SHM, $$$ $p < 0.001$ vs. SHM. CON: control group; SHM: sham cold water immersion group; STR PL: cold water immersion group supplemented with placebo; STR SUP: cold water immersion group supplemented with vitamin D_3.

3.2. Vitamin D Biochemical Analysis

3.2.1. Plasma Vitamin D_3 Metabolite Levels in DEX-Treated Rats

After four weeks of the experiment, the level of 25(OH)D_3 significantly differed between the groups. As we expected, the highest concentration of 25(OH)D_3 was observed in the supplemented group. However, the dexamethasone-treated supplemented with vitamin D_3 (DEX SUP) group differed only from the dexamethasone-treated placebo (DEX PL) group, not from the CON group (the values were 12.59 ± 2.87 in the DEX SUP, 5.87 ± 1.62 in the DEX PL, and 9.85 ± 4.12 ng/mL in the CON groups, respectively) (Figure 2B).

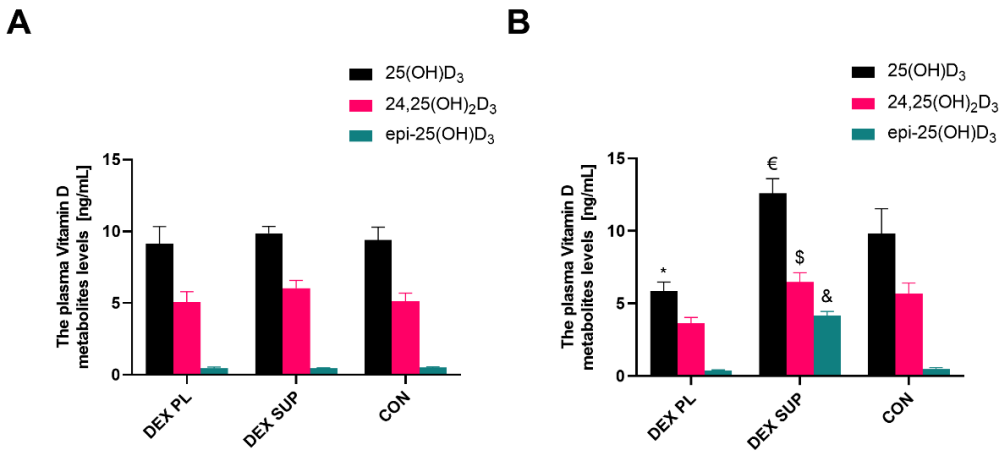

Figure 2. The plasma vitamin D metabolite levels in DEX-treated rats at baseline (**A**) and the end of the experiment (**B**). Results are expressed as mean ± SEM. DEX PL (n = 7), DEX SUP (n = 8), CON (n = 6), * $p < 0.05$ vs. CON, $ $p < 0.01$ vs. DEX PL, € $p < 0.001$ vs. DEX PL, & $p < 0.0001$ vs. DEX PL; vs. CON. DEX PL: dexamethasone-treated group supplemented with placebo; DEX SUP: dexamethasone-treated group supplemented with vitamin D_3; CON: control group.

In the CON group within the experiment, the results were relatively homogeneous and transparent. No changes were observed in either 25(OH)D_3 or 24,25$(OH)_2D_3$ during the experiment, proving the correct blinding in the group and the lack of vitamins D_3 or D_2 in the feed.

In the DEX PL group we observed a significant reduction in the bioavailable form of vitamin D_3-25(OH)D_3. Besides, no catabolic mechanisms were activated: the values for epi-25(OH)D_3 and 24,25$(OH)_2D_3$ were lower, but their ratio to 25(OH)D_3 remained practically unchanged between time points. This could reflect a clinical case of high demand for vitamin D_3 and its heavy consumption to defend muscles against atrophy. Circulating 25(OH)D_3 is absorbed first. The rapid mobilization of vitamin D_3 from the body's fat reserves is not visible here. The appearance of a large pool of endogenous vitamin D_3 would be manifested in changes in the ratio of 25(OH)D_3 to epi-25(OH)D_3 and 24,25$(OH)_2D_3$, respectively (as in the DEX SUP group, where vitamin D_3 was obtained exogenously) (Table 1).

Table 1. The 25(OH)D_3 and its metabolite ratios at baseline and the end of the experiment in DEX-treated rats.

Group	25(OH)D_3: 24,25$(OH)_2D_3$ Ratio		25(OH)D_3: epi-25(OH)D_3 Ratio	
	Baseline	after	Baseline	after
DEX PL (n = 7)	1.83 ± 0.18	1.65 ± 0.30	20.27 ± 3.08	16.04 ± 2.51 *
DEX SUP (n = 8)	1.70 ± 0.40	2.17 ± 1.00	21.97 ± 3.22	3.11 ± 0.88 #
CON (n = 6)	1.88 ± 0.48	1.68 ± 0.30	20.33 ± 6.17	20.62 ± 4.63

Results are expressed as mean ± SD. * $p < 0.05$ vs. CON, # $p < 0.0001$ vs. DEX PL; vs. CON.

Additionally, the lack of an increase in 24,25$(OH)_2D_3$ and significant changes in the 25(OH)D_3:24,25$(OH)_2D_3$ ratio, which works on the principle of feedback with 1,25$(OH)_2D_3$, supports the thesis that a rapid "on-going" consumption of bioavailable vitamin D_3 to protect against muscle atrophy in that particular group occurred (Table 1).

3.2.2. Plasma Vitamin D_3 Metabolite Levels in Stressed Rats

Similar to the DEX-treated rats, a significantly higher concentration of 25(OH)D_3 was observed only in supplemented group (22.89 ± 6.02) as compared with the STR PL (10.36 ± 2.92) and the SHM groups (7.84 ± 2.80 ng/mL). In addition, there was no effect of warm water immersion on native vitamin D_3 concentration and metabolism. Additionally,

in the STR PL group, the results were the same as for the SHM groups. This also means that cold water immersion had no effect on native vitamin D_3 concentration and metabolism (Figure 3B).

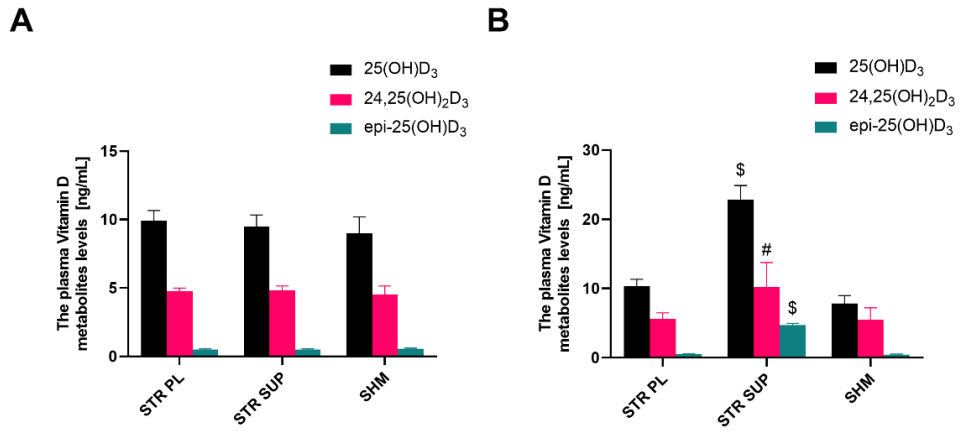

Figure 3. The plasma vitamin D metabolite levels in stressed rats at baseline (**A**) and the end of the experiment (**B**). Results are expressed as mean ± SEM. STR PL (n = 9), STR SUP (n = 9), SHM (n = 6), # $p < 0.01$ vs. STR PL; vs. SHM, $ $p < 0.0001$ vs. STR PL; vs. SHM. STR PL: cold water immersion group supplemented with placebo; STR SUP: cold water immersion group supplemented with vitamin D_3; SHM: sham cold water immersion group.

Considering the above, the STR SUP group may be treated as a positive control of vitamin D_3 supplementation. A fully statistically significant increase in both 25(OH)D_3, epi-25(OH)D_3, and 24,25$(OH)_2D_3$ shows that the body responds correctly to the supplementation. A particularly clear difference is visible for epi-25(OH)D_3, and the 25(OH)D_3:epi-25(OH)D_3 ratio. It is worth noting that in rodents (unlike humans), epimerization is the main "default" path of catabolism in response to exogenous vitamin D_3 (Table 2).

Table 2. The 25(OH)D_3 and its metabolite ratios at baseline and the end of the experiment in stressed rats.

Group	25(OH)D_3: 24,25$(OH)_2D_3$ Ratio		25(OH)D_3: epi-25(OH)D_3 Ratio	
	Baseline	**after**	**Baseline**	**after**
STR PL (n = 9)	2.10 ± 0.40	1.82 ± 0.36	20.20 ± 3.21	20.63 ± 1.75
STR SUP (n = 9)	1.94 ± 0.23	2.43 ± 0.92 &	19.34 ± 2.71	4.96 ± 1.34 $
SHM (n = 6)	1.99 ± 0.22	1.40 ± 0.21	16.88 ± 3.79	17.25 ± 4.99

Results are expressed as mean ± SD, & $p < 0.01$ vs. SHM, $ $p < 0.0001$ vs. STR PL; vs. SHM.

3.3. Morphological Analysis

3.3.1. Body and Skeletal Muscle Mass in DEX-Treated Rats

The DEX-treated groups presented weight loss throughout the experiment as compared with the CON group (Figure 4A).

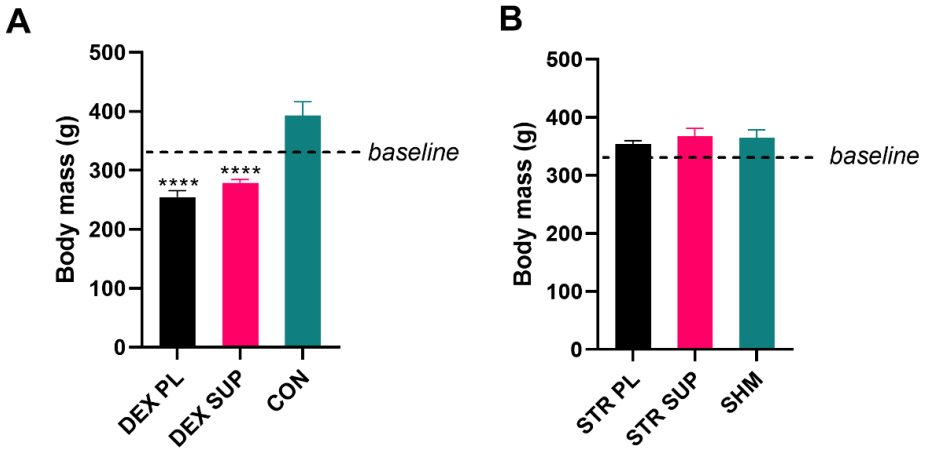

Figure 4. The body mass at the end of the experiment in DEX-treated (**A**), and stressed (**B**) rats. Results are expressed as mean ± SEM. DEX PL (n = 7), DEX SUP (n = 8), CON (n = 6), STR PL (n = 9), STR SUP (n = 9), SHM (n = 6), **** $p < 0.0001$ vs. CON. DEX PL: dexamethasone-treated group supplemented with placebo; DEX SUP: dexamethasone-treated group supplemented with vitamin D_3; CON: control group; STR PL: cold water immersion group supplemented with placebo; STR SUP: cold water immersion group supplemented with vitamin D_3; SHM: sham cold water immersion group.

To investigate skeletal muscle loss, the weights of the soleus (SOL), extensor digitorum longus (EDL) (Figure 5A,B), tibialis anterior, gastrocnemius, and quadriceps femoris muscles were measured immediately after excision (Table 3). Although supplementation with vitamin D_3 did not prevent this DEX-induced body mass loss, we found statistically significant differences in SOL muscle mass between the DEX PL and the DEX SUP groups (122 ± 15, and 149 ± 9 mg, respectively; $p < 0.05$). Additionally, there was no difference between the DEX SUP and the CON group (Figure 5A).

Table 3. Body and skeletal muscles mass at the end of the experiment in DEX-treated rats.

Group	Basal Body Mass (g)	Final Body Mass (g)	Tibialis Anterior (g)	Gastrocnemius (g)	Quadriceps Femoris (g)
DEX PL (n = 7)	332.57 ± 23.62	254.86 ± 28.06 $	0.39 ± 0.11 $	1.28 ± 0.20 $	1.41 ± 0.24 $
DEX SUP (n = 8)	337.38 ± 24.54	279.13 ± 14.55 $	0.46 ± 0.04 $	1.42 ± 0.16 $	1.58 ± 0.26 $
CON (n = 6)	335.60 ± 48.20	393.00 ± 52.05	0.75 ± 0.05	2.24 ± 0.18	2.44 ± 0.29

Results are expressed as mean ± SD. $ $p < 0.0001$ vs. CON.

Regarding the EDL, we observed a significant reduction in muscle mass in both DEX-treated groups compared with the CON group (107 ± 19 DEX PL, 113 ± 13 DEX SUP, and 161 ± 21 mg CON, respectively; $p < 0.001$). Nevertheless, there were no differences between the supplemented and placebo groups. These results suggest that vitamin D_3 supplementation influences DEX-induced muscle loss, but only in red, not white muscle, and may preserve red muscle against the chronic DEX-induced muscle loss. The masses of the other muscles were consistent and homogeneous. There was a clear and highly statistically significant reduction in all muscle mass in both DEX-treated groups regardless of supplementation with vitamin D_3 or placebo.

No significant difference was found in the muscle:body weight ratios of CON and DEX-treated rats in EDL (Figure 6B). However, the SOL muscle weight to body weight ratio significantly increased in DEX-treated rats compared to the CON group (Figure 6A). This showed not only that there was a relative sparing of SOL muscles as compared with EDL within the experiment, but also sparing of muscle tissue in general relative to other body components. In particular, SOL sparing affected the DEX SUP group, suggesting a protective role of vitamin D_3 supplementation.

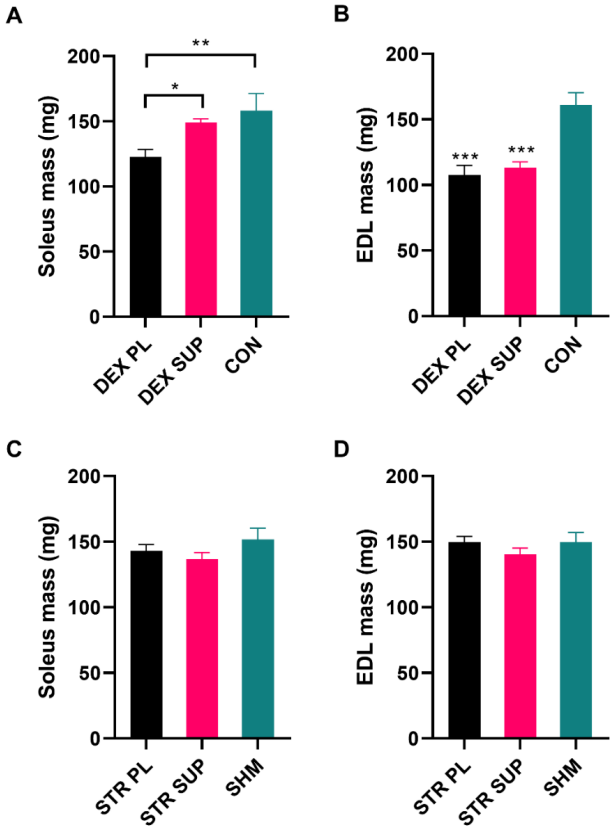

Figure 5. The SOL and EDL muscles mass at the end of the experiment in DEX-treated (**A**,**B**), and stressed (**C**,**D**) rats. Results are expressed as mean ± SEM. DEX PL (n = 7), DEX SUP (n = 8), CON (n = 6), STR PL (n = 9), STR SUP (n = 9), SHM (n = 6), * $p < 0.05$ vs. DEX SUP, ** $p < 0.01$, *** $p < 0.001$ vs. CON. DEX PL: dexamethasone-treated group supplemented with placebo; DEX SUP: dexamethasone-treated group supplemented with vitamin D_3; CON: control group; STR PL: cold water immersion group supplemented with placebo; STR SUP: cold water immersion group supplemented with vitamin D_3; SHM: sham cold water immersion group.

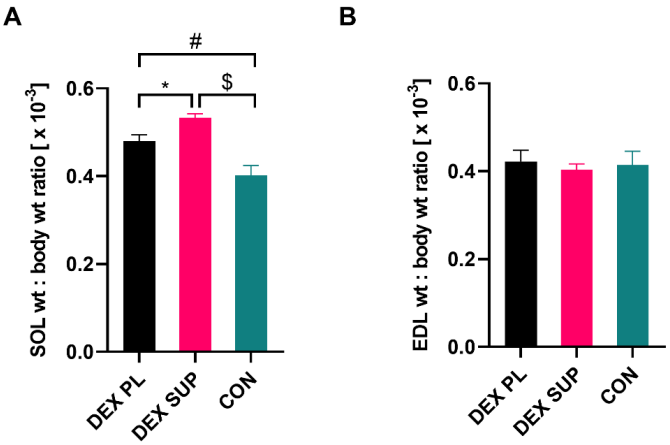

Figure 6. Mean muscle weight: body weights ratio in DEX-treated rats in SOL (**A**), and EDL (**B**). Results are expressed as mean ± SEM. DEX PL (n = 7), DEX SUP (n = 8), CON (n = 6), * $p < 0.05$, # $p < 0.01$, $ $p < 0.0001$. DEX PL: dexamethasone-treated group supplemented with placebo; DEX SUP: dexamethasone-treated group supplemented with vitamin D_3; CON: control group; EDL: extensor digitorum longus; SOL: soleus.

3.3.2. Body and Skeletal Muscle Mass in Stressed Rats

Interestingly, despite highly statistically significant CORT release (Figure 1), we did not observe body weight changes in the group of rats subjected to cold water immersion (Figure 4B). Furthermore, no statistically significant differences were observed between the groups in SOL (143 ± 15, 136 ± 16, and 152 ± 21 mg in the STR PL, STR SUP, and SHM groups, respectively) and EDL (STR PL 149 ± 13, STR SUP 140 ± 15, SHM 149 ± 18 mg) muscle mass (Figure 5C,D).

Although we did not observe any statistically significant differences between groups in stressed rats with regard to muscle mass (Table 4), as opposed to the DEX SUP group, stressed rats supplemented with vitamin D_3 had the lowest (statistically insignificant) ratios in both muscles among groups (Table 5). Considering the lack of body reduction in stressed groups, that result supports the thesis that the used stress model promotes adipose tissue gain (in turn, an enlarged adipose mass may serve as a reservoir for vitamin D).

Table 4. Body and skeletal muscle mass at the end of the experiment in stressed rats.

Group	Basal Body Mass (g)	Final Body Mass (g)	Tibialis Anterior (g)	Gastrocnemius (g)	Quadriceps Femoris (g)
STR PL (n = 9)	324.44 ± 22.10	353.89 ± 17.32	0.69 ± 0.05	2.10 ± 0.16	2.71 ± 0.29
STR SUP (n = 9)	337.78 ± 41.77	367.67 ± 40.43	0.67 ± 0.07	2.15 ± 0.25	2.70 ± 0.45
SHM (n = 6)	329.00 ± 33.66	364.83 ± 33.52	0.71 ± 0.07	2.16 ± 0.33	2.79 ± 0.48

Results are expressed as mean ± SD.

Table 5. Mean muscle weight: body weights ratio in stressed rats.

Group	SOL Weight: Body Weight Ratio ($\times 10^{-3}$)	EDL Weight: Body Weight Ratio ($\times 10^{-3}$)
STR PL (n = 9)	0.403 ± 0.036	0.423 ± 0.039
STR SUP (n = 9)	0.374 ± 0.048	0.383 ± 0.038
SHM (n = 6)	0.415 ± 0.035	0.410 ± 0.050

Results are expressed as mean ± SD.

3.3.3. Internal Organ Mass in DEX-Treated Rats

Heart and liver weights did not differ significantly between groups. Nevertheless, differences in the weight of the organs of the lymphatic system were observed. Thus, spleen weight was the lowest of the two DEX-treated groups. Besides, there was a statistically significant reduction in thymus weight in both DEX-treated groups compared to the control group (0.090 ± 0.053, 0.148 ± 0.091 vs. 0.372 ± 0.054 g in the DEX PL, DEX SUP, and CON group, respectively) (Table 6).

Table 6. Internal organ mass at the end of the experiment in DEX-treated rats.

Group	Heart (g)	Liver (g)	Spleen (g)	Thymus (g)
DEX PL (n = 7)	0.83 ± 0.11	10.26 ± 0.93	0.25 ± 0.04 $	0.090 ± 0.053 $
DEX SUP (n = 8)	0.92 ± 0.09	11.08 ± 1.34	0.30 ± 0.03 $	0.148 ± 0.091 #
CON (n = 6)	0.92 ± 0.10	11.88 ± 2.16	0.65 ± 0.06	0.372 ± 0.054

Results are expressed as mean ± SD, # $p < 0.001$ vs. CON, $ $p < 0.0001$ vs. CON.

3.3.4. Internal Organs Mass in Stressed Rats

Similarly to DEX-treated groups, in stressed rats we did not observe any statistically significant changes in heart and liver mass. Nevertheless, we observed significantly lower thymus weight in the stressed animals treated with placebo, but not in the vitamin D_3-supplemented group compared with the SHM group (0.233 ± 0.045 in the STR PL, 0.305 ± 0.101 in the STR SUP, and 0.348 ± 0.082 g in the SHM groups, respectively; $p < 0.05$), which suggest that vitamin D_3 supplementation may protect thymus against degeneration caused by GCs, particularly in the chronic stress condition (Table 7).

Table 7. Internal organs mass at the end of the experiment in stressed rats.

Group	Heart (g)	Liver (g)	Spleen (g)	Thymus (g)
STR PL (n = 9)	0.94 ± 0.05	11.22 ± 0.77	0.63 ± 0.08	0.233 ± 0.045 *
STR SUP (n = 9)	0.95 ± 0.06	10.77 ± 1.31	0.66 ± 0.07	0.305 ± 0.101
SHM (n = 6)	0.89 ± 0.09	11.48 ± 1.46	0.60 ± 0.11	0.348 ± 0.082

Results are expressed as mean ± SD. * $p < 0.05$ vs. SHM.

4. Discussion

The role of vitamin D_3 within the skeletal muscle is in the scope of interest of many researchers. Although the beneficial effect of vitamin D_3 on skeletal muscle mass remains unclear, some research suggests that vitamin D_3 may prevent skeletal muscle loss and atrophy [24], while other papers indicate that vitamin D_3 supplementation has little or no effect on muscle mass [12,13]. We found that chronic DEX treatment decreased serum 25(OH)D_3 concentrations. We also showed that GC-induced body and muscle loss are presented only in exogenously administered DEX. Our results indicate that DEX-induced muscle loss is abolished in rats supplemented with vitamin D_3 but only in SOL muscle. Based on previously published data [25], we assumed that a similar effect should be observed in the CSR conditions. However, instead of HPA axis activation and CORT surge, we did not note any changes in the body and muscle weight. Moreover, the cold water immersion had no effect on the native vitamin D_3 levels despite the highly and statistically significant elevated level of circulating CORT in stressed rats.

4.1. Chronic Stress Response and Its Effect on the Body, Skeletal Muscle, and Organ Mass

The chronic stress response model used in the experiment was based on the procedure that combines physical (low temperature) and psychological stress (impossibility to escape and isolation). The obtained data show that the CSR was successfully induced, and the level of circulating CORT level highly increased from the baseline compared to the SHM and CON groups. The CORT concentration at 400 ng/mL levels corresponded with the results obtained in our other CSR experiment [26] and with the works based on the cold water immersion model [27,28].

Despite the physiologically significant CORT surge, we did not observe any body and muscle weight changes in the supplemented and placebo groups. Interestingly, in an experiment conducted by Nishida and coworkers, no changes in SOL and EDL muscle mass were observed even during DEX treatment (5 days, dose 600 μg/kg) [29]. Our recent work [25,26] showed that the CSR and increased CORT level do not have to accompany changes in body and muscle weight (data not shown); nevertheless, an increased level of atrogin-1 was observed. Furthermore, we assume that to explain this phenomenon in these particular conditions, it is necessary to consider intramuscular fat stores in rats from groups exposed to the chronic cold water immersion [30,31]. In support of this thesis, we can mention that, during autopsy, an increased amount of total fat mass and adipose tissue browning in two reference points (the suprascapular and supraspinal areas) was observed [32] (data not shown).

Lastly, the classically indicated "stress triad" (a term proposed by Selye) assumes that in first reaction to stress there are three visible changes: adrenal enlargement, atrophy of the thymus, and hemorrhagic gastric erosions [33]. A partly similar observation was made in our study, where thymus weight significantly decreased in rats from the STR PL, while no changes in the STR SUP group were observed (Table 7).

4.2. Dexamethasone Treatment and Its Effect on the Body, Skeletal Muscle, and Organ Mass

Our results show that chronic DEX administration reduced body weight in both DEX-treated groups (23% and 17% in DEX PL and DEX SUP, respectively), and supplementation with vitamin D_3 did not attenuate this effect in a statistically significant manner. Moreover, in line with Selye's assumptions, thymus and spleen degradation was also observed in both DEX-treated groups. Despite the lack of bodyweight preservation in the DEX SUP group,

SOL muscle-sparing in the vitamin D_3 supplemented group was observed. Muscle atrophy is a major adverse effect observed after DEX administration; however, the exact mechanism responsible for DEX-induced muscle atrophy is not well understood. Some data show that DEX acts mainly on muscles containing type II fast-twitch fibers as compared with type I slow-twitch fibers [34,35]. Similar observations were made by Krug and coworkers, where DEX treatment reduced flexor hallucis longus and tibialis anterior mass without SOL mass loss [36]. Our results are only partially in line with previous observations. Chronic DEX administration caused massive muscle loss in both red and white muscles, sparing only SOL in the group supplemented with vitamin D_3.

4.3. The Potential Protective Role of Vitamin D_3 in Skeletal Muscle in GC-Induced Muscle Loss Conditions

Our results show the massive consumption of vitamin D_3 in DEX-treated rats to defend muscles against atrophy, especially in the DEX PL group. Circulating 25(OH)D_3 was utilized first without the rapid mobilization of vitamin D_3 from the body's fat reserves. Furthermore, in the DEX SUP group, the rapid "on-going" consumption of circulating vitamin D_3 to protect against muscle atrophy was visible.

Several clinical works highlight the positive aspects of vitamin D supplementation in many diseases. According to the Endocrine Society Clinical Practice Guideline on the Prevention of Vitamin D Deficiency, concentrations of 25(OH)D_3 from 21 to 29 ng/mL (52.5–72.5 nmol/L) in serum are insufficient, and levels lower than 20 ng/mL (50 nmol/L) are considered to reflect deficiency [37]. As the data show, normalizing the level of circulating vitamin D enhances the reduction of systemic inflammation markers and intensity of pain in low back patients [37]. Another study shows that supplementation for six months reduced oxidative protein damage, decreased pain, improved quality of life, and improved grip strength and physical performance in osteoarthritis patients [38]. Vitamin D supplementation is also increasingly used in the prevention and therapy of sarcopenia [5,6] and neuromuscular diseases [39,40]. Moreover, studies show that vitamin D deficiency results in a more severe course of SARS-COV2 virus infection, and vitamin D supplementation is one of the proposed strategies for relieving symptoms of the disease [41]. Additionally, novel findings suggest that the early use of DEX could reduce duration of mechanical ventilation and overall mortality in patients with established moderate-to-severe acute respiratory distress syndrome (ARDS) in SARS-COV2 infected patients [42]. The low potential risk of vitamin D overdose (using the standard proposed dose appropriate to age, e.g., 2000 IU for an adult) [43,44], and the cost-effective aspect of vitamin D supplementation [45] should be considered in support of its use for the treatment of various diseases. According to knowledge about several common regulatory pathways which vitamin D and DEX share [46], using these therapeutics in combination may prove to be the most effective known strategy against SARS-COV and also other diseases (i.e., rheumatoid arthritis [22]) due to improved function of the immune system and minimized side effects of DEX-treatment.

However, the mechanism of vitamin D-mediated changes in skeletal muscle is not fully elucidated. It is known that vitamin D_3 acts mainly via specific binding to an intracellular VDR, interacting with specific nucleotide sequences of over 60 target genes [47]. Numerous data show that the interaction between GCs and VDR occurs. Therefore, in the study conducted by Hidalgo and coworkers, induction with GCs increased VDR transcription in squamous cell carcinoma VII (SCC) to the level of 4–6 fold higher compared to the control group [48].

Additionally, a novel mitochondrial localization of VDR has been described. Data show that VDR influences mitochondrial respiration reduction and serves in reprogramming in cell metabolism toward the biosynthetic pathways [49,50]. This underlines the importance of mitochondria as the hub linking the processes such as cell development and atrophy inhibition in skeletal muscles. Other studies suggest that VDR plays a fundamental regulatory role in skeletal muscle mitochondrial function [51]. Moreover, the cooperative action of vitamin D_3 and GCs in modulating gene expression was presented [17] which

implies the potential reduction of the adverse effects of GC excess (during vitamin D_3 supplementation) [52]. In our study, this phenomenon is partly confirmed because SOL muscle consists of predominantly slow-oxidative fibers, with a larger pool of mitochondria, and EDL is mainly formed of fast-glycolytic muscle fibers [53]. Our results show that in DEX-induced atrophy rats, SOL muscle is sparing in both absolute (Figure 5A) and relative values (Figure 6A). In summary, the main explanation for such a massive decrease in vitamin D concentration with partial protection against atrophy is the supposition that skeletal muscle cells overexpress VDR under both atrophy and hypertrophy conditions [54].

5. Conclusions

Our findings show that despite the elevated circulating CORT in cold water-immersed rats, no body and muscle weight changes were observed in either the vitamin D_3-supplemented or placebo groups. We found that chronic DEX treatment decreased serum 25(OH)D_3 concentrations, and cold water immersion had no effect on native vitamin D_3 levels. Moreover, body weight and muscle loss occurred concomitantly only with exogenously administered DEX. Our results indicate that DEX-induced muscle loss was abolished in rats supplemented with vitamin D_3, but only concerning the SOL muscle. The massive consumption of endogenous vitamin D_3 was caused by an attempt to protect against muscle loss in DEX-treated rats. The additional supply of exogenous vitamin D_3 in the DEX SUP group supports that this rapid "on-going" utilization of circulating vitamin D_3 was accompanied by the protection of muscle atrophy. Our findings show that DEX treatment should be combined with vitamin D_3 supplementation since the long-term treatment of DEX leads to a sharp reduction in vitamin D_3 levels. Moreover, as a consequence, this may contribute to the adverse effects of DEX treatment alone.

Study Limitation

The findings of the present study are limited because the experiments were focused on visible morphological changes and not on the mechanism(s) responsible for the effects of vitamin D_3 supplementation on GC-induced muscle atrophy per se. Nevertheless, we found that supplementation with vitamin D_3 reduced the adverse effects on muscle loss in chronic DEX-treated rats, which indicates that further studies are needed to clarify the possible molecular mechanism(s) explaining this phenomenon.

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Abbreviations

CORT	Corticosterone
CSR	Chronic stress response
DEX	Dexamethasone
FOXO-1	Forkhead box protein O1
GCs	Glucocorticoids
HPA	Hypothalamic–pituitary–adrenal axis
HSD11B1	11β–hydroxysteroid dehydrogenase type 1
VDR	Vitamin D receptor
1,25–D3-MARRS	1,25D3–membrane associated, rapid response steroid–binding

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