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Wpływ melatoniny i resweratrolu na zmiany struktury i funkcji dehydrogenazy aldehydu 3-fosfoglicerynowego i dehydrogenazy mleczanowej indukowane reaktywnymi formami tlenu i azotu

The influence of melatonin and resveratrol on the structural and functional changes of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase induced by reactive oxygen and nitrogen species

Praca doktorska

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Spis treści

1 Podstawowe informacje o pracy doktorskiej

Niniejsza rozprawa doktorska stanowi cykl trzech spójnych tematycznie publikacji naukowych. Poniżej podano wykaz tych publikacji wraz z numeracją, której kolejność przyjęto odnosząc się do prac w dalszych częściach opracowania.

1. Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483. (IF 2,368, 70 pkt. MNiSW)
2. Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885. (IF 3.681, 35 pkt. MNiSW)
3. Rodacka, A., Strumillo, J., Serafin, E., Puchala, M. (2015). Analysis of Potential Binding Sites of 3,5,4'-Trihydroxystilbene (Resveratrol) and trans-3,3',5,5'-Tetrahydroxy-4'-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols. *Journal of Physical Chemistry B*, *119*(30), 9592-9600. (IF 3,187, 30 pkt. MNiSW).

Sumaryczna wartość IF prac wchodzących w skład doktoratu wynosi: 9,236

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1. Narodowego Centrum Nauki w ramach projektu Opus nr 2012/05/B/NZ1/00701 pt. „Badanie indukowanych przez reaktywne formy tlenu i azotu procesów agregacji GAPDH i innych białek biorących udział w chorobach neurodegeneracyjnych. Ocena antyoksydantów w przeciwdziałaniu tym procesom”.
2. Ministerstwa Nauki i Szkolnictwa Wyższego w ramach dotacji badań naukowych młodych naukowców w latach: 2015-2016.

2 Wprowadzenie teoretyczne

W większości chorób związanych z wiekiem stres oksydacyjny i nitrozacyjny jest uznawany za jedną z przyczyn lub konsekwencję powstania choroby. Przykładem takich chorób są choroby neurodegeneracyjne, a wśród nich choroba Alzheimera (AD), Parkinsona (PD), Huntingtona (HD) czy stwardnienie zanikowe boczne (ALS) (Andersen, 2004; Collin, 2019; Nakamura i Lipton, 2017). Z wyjątkiem odmian rodzinnych tych schorzeń większość z nich dotyka osoby po 65 roku życia.

Nasilający się wraz z wiekiem lub w czasie choroby stres oksydacyjny jest wynikiem zaburzenia równowagi między ilością powstających w komórce tzw. reaktywnych form, w tym tlenu i azotu (RFT i RFA), a możliwościami ich usuwania przez cząsteczki lub enzymy o właściwościach antyoksydacyjnych. Przeważają wtedy procesy uszkodzenia komórkowych makrocząsteczek przez RFT i RFA (dystres oksydacyjny), nad procesami związanymi z przekazywaniem sygnału redoks (eustres oksydacyjny) (Moldogazieva i in., 2018; Sies i in., 2017; Wall i in., 2012).

Podstawowym, endogennym źródłem RFT jest oddychanie tlenowe. Neurony polegają głównie na tlenowym metabolizmie glukozy, zużywając około 25% dostarczanej do organizmu glukozy i 20% dostarczanego tlenu, z tego powodu są bardziej narażone na zaburzenia równowagi redoks i stres oksydacyjny (Nakamura i Lipton, 2017). Podczas wytwarzania energii w warunkach tlenowych, niepełna redukcja tlenu przez elektrony mitochondrialnego łańcucha oddechowego jest przyczyną powstania anionorodnika ponadtlenkowego ($O_2^{\cdot -}$), który ulega dysmutacji do nadtlenku wodoru (H_2O_2). Mitochondria są zarówno głównym producentem jak i głównym celem RFT, co więcej gromadzenie oksydacyjnych uszkodzeń białek mitochondrialnych nasila produkcję RFT. Oprócz mitochondriów, także duża zawartość jonów metali (Fe^{2+}, Cu^{2+}) w ośrodkowym układzie nerwowym (OUN) sprzyja generacji najbardziej reaktywnej formy tlenu, czyli rodnika wodorotlenowego ($^{\cdot}OH$) w reakcji Fentona i Habera-Weissa (Cheignon i in., 2018; Collin, 2019).

Źródłem reaktywnych form azotu (RFA) są natomiast obecne w neuronach syntazy tlenku azotu: neuronalna (nNOS, NOS1) i indukowalna (iNOS, NOS2). Enzymy te produkują tlenek azotu (NO) na drodze przemiany aminokwasu argininy w cytrulinę z udziałem NADPH. W warunkach fizjologicznych NO odpowiada za procesy takie jak zapamiętywanie, przekaźnictwo nerwowe (Nakamura i in., 2015). Wraz z wiekiem, a także wskutek działania toksyn środowiskowych lub niektórych leków dochodzi do

nadmiernej produkcji NO z udziałem iNOS oraz nNOS, co przyczynia się do powstania stresu nitrozacyjnego (Nakamura i Lipton, 2017). Ponadto, tlenek azotu w reakcji z tlenem daje szereg innych RFA, w tym dwutlenek azotu (NO_2), trójtlenek azotu (N_2O_3), a w reakcji z anionorodnikiem ponadtlenkowym wysoce toksyczny nadtlenoazotyn ($ONOO^-$).

Celem dla RFT i RFA w komórce są wszystkie najważniejsze makrocząsteczki w tym, lipidy, kwasy nukleinowe, węglowodany oraz białka. Oksydacyjne modyfikacje białek są uznawane jako swoisty marker w przebiegu wyżej wymienionych chorób neurodegeneracyjnych, a ich konsekwencje są jedną z przyczyn dysfunkcji i śmierci komórek nerwowych (Reeg i Grune, 2015; Sies i in., 2017). Wśród oksydacyjnie zmienionych białek w przebiegu tych chorób, można znaleźć białka pełniące wiele odmiennych funkcji, w tym białka glikolityczne (GAPDH, LDH), mitochondrialne, cytoszkieletu, opiekuńcze oraz systemu ubikwityna-proteasom (Butterfield i Boyd-Kimball, 2018; Reeg i Grune, 2015). Konsekwencją zmian funkcjonalnych i strukturalnych w wymienionych białkach jest między innymi, nasilenie istniejącego stresu oksydacyjnego, zaburzenie wytwarzania energii, uszkodzenie mitochondriów oraz zaburzenie proteostazy i gromadzenie agregatów białkowych zewnątrz- lub wewnątrzkomórkowo.

Badanie skutków oksydacyjnych modyfikacji i uszkodzeń białek w przebiegu chorób neurodegeneracyjnych jest jednym ze sposobów zrozumienia, ale także poszukiwania skutecznych metod zapobiegania i leczenia, tych jak dotąd nieuleczalnych chorób.

Szczególne miejsce w patomechanizmie chorób neurodegeneracyjnych zajmuje dehydrogenaza aldehydu 3-fosfoglicerynowego (GAPDH) (Avila i in., 2017; Butterfield i in., 2010; Muronetz i in., 2017). Jest to enzym glikolityczny, zaangażowany w cykl reakcji przemiany glukozy w pirogronian, katalizujący oksydatywną fosforylację aldehydu 3-fosfoglicerynowego w 1,3-bisfosfoglicerynian. GAPDH jest uważane za sensor stresu oksydacyjnego, gdyż bardzo łatwo ulega odwracalnym i nieodwracalnym modyfikacjom oksydacyjnym (Hara i in., 2006; Hildebrandt i in., 2015; Zaffagnini i in., 2013).

Jedne z pierwszych badań nad udziałem GAPDH w śmierci neuronów, dotyczyły translokacji S-nitrozylowanego enzymu (GAPDH-SNO) do jądra komórkowego wraz z ligazą ubikwityny Siah-1, co uruchamiało szlak sygnalizacyjny prowadzący do apoptozy (Hara i in., 2005). Późniejsze badania pokazały, iż w jądrze komórkowym

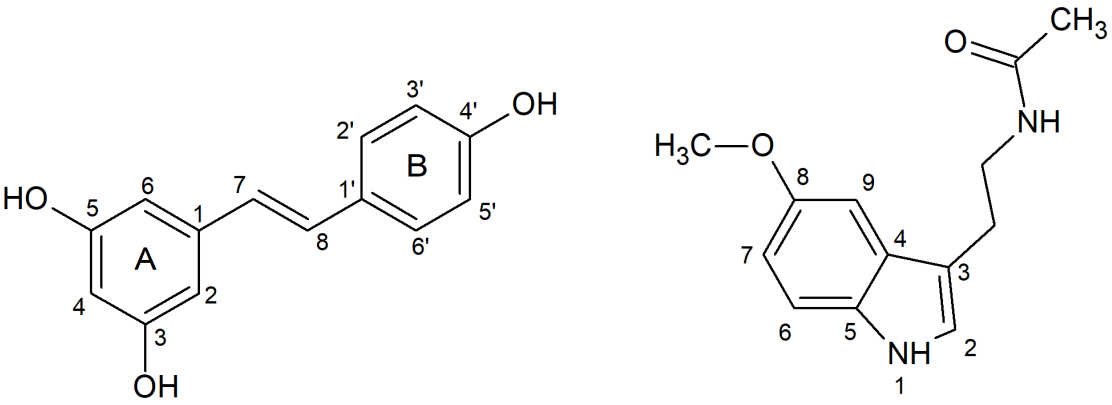
GAPDH pełni rolę transnitrozylazy, przenoszącej sygnał związany z NO na inne białka, także w warunkach fizjologicznych (Kornberg i in., 2010). Obecnie wiadomo, iż wykonywana z udziałem GAPDH-SNO transnitrozylacja jądrowych białek, w tym deacetylazy SIRT1 oraz aktywacja acetylotransferazy p300, jest jednym z mechanizmów zwiększonego poziomu acetylowanego białka tau. Podobnie jak hiperfosforylacja, modyfikacja ta jest odpowiedzialna za agregację tau w przebiegu AD (Sen i in., 2018).

Śmierć komórek nerwowych z udziałem GAPDH może odbywać się również poza szlakiem związanym z translokacją do jądra komórkowego. W odpowiedzi na niski poziom stresu oksydacyjnego, GAPDH lokalizuje się w rejonie błony mitochondrialnej, gdzie uczestniczy w mikroautofagii (mitofagii) uszkodzonych oksydacyjnie mitochondriów (Butera i in., 2019). Jest to mechanizm adaptacyjny, zapobiegający śmierci komórek, jednak wskutek oddziaływania ze zmutowaną huntingtyną (mHtt) w przebiegu choroby Huntingtona, ta aktywność zostaje zahamowana, co prowadzi do apoptozy (Hwang i in., 2015). Ponadto nadmiar RFT i RFA może powodować powstanie wewnątrzkomórkowych, amyloidowych agregatów GAPDH (Nakajima i in. 2009). Wykazano, iż agregaty te powodują śmierć nekrotyczną lub apoptozę związaną ze wzrostem przepuszczalności megakanału mitochondrialnego PTP (ang. permeability transition pore) oraz uwolnieniem czynników proapoptotycznych (Nakajima i in., 2017). Co więcej, badania Itakura i in. (2015) pokazały, iż agregaty oksydacyjnie zmienionej GAPDH mogą przyspieszać agregację peptydu amyloidu beta (Aβ40), czyniąc nowo powstałe agregaty wysoce toksycznymi dla komórek nerwowych w przebiegu AD.

Mnogość szlaków sygnalizacyjnych, lokalizacji komórkowych i interakcji z innymi białkami zaangażowanymi w patomechanizm chorób neurodegeneracyjnych oksydacyjnie zmienionej GAPDH powoduje, iż badanie modyfikacji tego białka z udziałem RFT i RFA jest wciąż aktualnym zagadnieniem.

Wraz z wiekiem oraz w przebiegu chorób w OUN spada aktywność kluczowych enzymów antyoksydacyjnych, takich jak dysmutaza ponadtlenkowa (SOD), katalaza (CAT), peroksydaza glutationowa (GPx) i reduktaza glutationowa (GR). Co więcej, zmniejsza się ilość drobnocząsteczkowych przeciwutleniaczy, w tym glutationu (GSH) (Andersen, 2004). Dlatego ważne jest poszukiwanie związków, które mogłyby stanowić wsparcie dla endogennego układu antyoksydantów. W niniejszej pracy podjęto próbę oceny dwóch naturalnie występujących przeciwutleniaczy w zapobieganiu oksydacyjnym modyfikacjom dehydrogenazy aldehydu 3-fosfoglicerynowego (GAPDH) i dehydrogenazy mleczanowej (LDH).

Do badań wybrano resweratrol (trans-3,5,4'-trihydroksystilben) - polifenol i fitoaleksynę oraz melatoninę (N-acetylo-5-metoksytryptamina) - pochodną indolu i niebiałkowy neurohormon (Ryc. 1).



trans-3,5,4'-trihydroksystilben
trans-Resweratol

N-acetylo-5-metoksytryptamina
Melatonina

Rycina 1. **Struktury chemiczne przeciwutleniaczy użytych w badaniach: resweratrolu i melatoniny.** Rycinę przygotowano z użyciem ACD/ChemSketch (Freeware), version 2019.1.2, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2019.

Oba antyoksydanty obecne są w wielu produktach pochodzenia naturalnego i mogą być przyjmowane z dietą (Meng i in., 2017; Salehi i in., 2018). Melatonina u ssaków jest produkowana przez szyszynkę, ale jest też wytwarzana przez rośliny, organizmy jednokomórkowe oraz syntetyzowana w mitochondriach (Reiter i in., 2018). Niestety, poziom syntezy melatoniny w OUN maleje wraz z wiekiem oraz w chorobach neurodegeneracyjnych, co dodatkowo potwierdza istotność stosowania diety bogatej w produkty zawierające ten przeciwutleniacz, czy też jego suplementację (Vincent, 2018).

Zarówno resweratrol jak i melatonina mają zdolność przekraczania bariery krew-mózg. Ich działanie neuroprotekcyjne wynika między innymi z neutralizacji RFT i RFA, chelatowania jonów metali, zwiększenia aktywności SOD, CAT, GPx, SIRT1 oraz zmniejszenia aktywności iNOS (Ansari Dezfouli i in., 2019; Boga i in., 2019; Rege i in., 2014; Reiter i in., 2018).

Oba związki w przebiegu chorób neurodegeneracyjnych regulują proces autofagii, wykazują działanie antyagregacyjne oraz wpływają na strukturę powstających białkowych agregatów, redukując ich neurotoksyczność (Cardinali, 2019; Ladiwala i in., 2010; Rege i in., 2014; Vincent, 2018). Co więcej, ostatnie badania pokazują, iż melatonina poprzez niekowalencyjne, hydrofobowe oddziaływania może doprowadzić

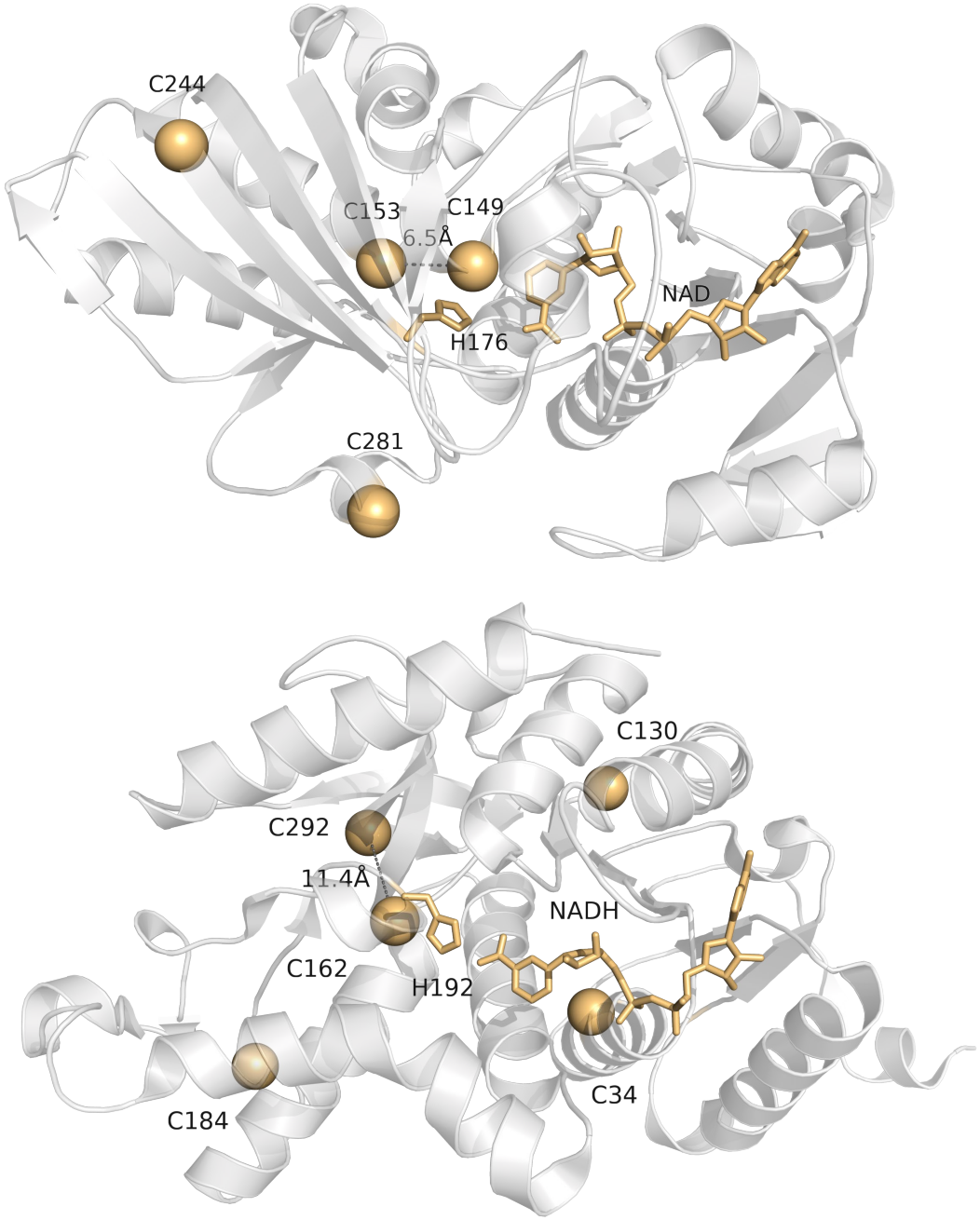
do dezintegracji dojrzałych agregatów białka tau, głównego składnika wewnątrzkomórkowych splątków neurofibrylarnych w przebiegu AD (Balmik i in., 2020).

3 Cel pracy

Celem pracy było określenie roli melatoniny i resweratrolu w oksydacyjnych uszkodzeniach dehydrogenazy aldehydu 3-fosfoglicerynowego (GAPDH) i dehydrogenazy mleczanowej (LDH). Dla realizacji celu pracy zbadano wpływ wybranych RFT i RFA takich jak: $^{\cdot}OH$, $O_2^{\cdot -}$, H_2O_2, NO na zmiany aktywności i struktury GAPDH i LDH. Następnie korzystając z uzyskanych wyników określono potencjał melatoniny i resweratrolu w zapobieganiu lub nasilaniu, oksydacyjnych modyfikacji i uszkodzeń w badanych białkach.

4 Główne założenia i zastosowane metody badawcze

W celu lepszego zrozumienia zmian strukturalnych i funkcjonalnych zachodzących w GAPDH pod wpływem RFT i RFA, zaplanowano badania porównujące wpływ tych reaktywnych form na tę dehydrogenazę oraz na enzym o podobnej strukturze, ale znacząco różnej budowie centrum aktywnego. Do badań oprócz GAPDH wybrano LDH, obie dehydrogenazy pochodzące z mięśni królika. Enzymy te w stanie natywnym, wykazują aktywność enzymatyczną jako białka tetrameryczne i pełnią funkcję NAD(H)-zależnych oksydoreduktaz. Mają podobną masę cząsteczkową, strukturę drugorzędową oraz zawartość najbardziej wrażliwych na oksydacyjne modyfikacje aminokwasów w tym reszt Cys, Met i Tyr. Największe różnice w budowie obu białek są zauważalne na poziomie ich centrum aktywnego (Ryc. 2).



Rycina 2. **Struktury podjednostek GAPDH (góra) i LDH (dół) w kompleksie z koenzymem NAD(H).** Na rycinie przedstawiono reszty cysteiny, aminokwasu ważnego z punktu widzenia oksydacyjnych modyfikacji. GAPDH posiada 4 reszty cysteiny, a LDH 5 reszt tego aminokwasu (lokalizację atomów węgla w pozycji α (Cα) reszt cysteinowych w podjednostkach zaznaczono za pomocą pomarańczowych kul, a odległości (Å) między najbliżej położonymi oznaczono przerywaną linią). Obszar centrum aktywnego wyznaczają: Cys-149, His-176 (GAPDH), H192 (LDH) oraz pierścień nikotynamidowy koenzymu NAD(H). Rycinę przygotowano z użyciem PyMOL Molecular Graphics System, Version 2.3.2, Schrödinger, LLC oraz wykorzystując struktury GAPDH i LDH (PDB ID 1J0X, 3H3F).

W przypadku GAPDH katalizowana reakcja przemiany aldehydu 3-fosfoglicerynowego (G-3-P) w 1,3-bisfosfoglicerynian (1,3-BPG) odbywa się z bezpośrednim udziałem Cys-149, His-176 oraz koenzymu NAD. Bliskie sąsiedztwo His-176 (~5 Å) powoduje obniżenie pKa reszty tiolowej Cys-149 z 8 do 5,9 i dysocjację w warunkach fizjologicznego pH z utworzeniem anionu tiolowego $Cys\text{-}S^-$ o właściwościach nukleofilowych (Didierjean i in., 2003). Dzięki tym właściwościom, Cys-149 w czasie katalizowanej reakcji tworzy kowalencyjny produkt pośredni tzw. hemitioacetal z węglem grupy karbonylowej G-3-P. Oprócz tworzenia pary jonowej z Cys-149, His-176 pełni rolę zasady, będącej akceptorem protonu w trakcie przeniesienia atomu wodoru z hemitioacetalu na cząsteczkę NAD i utworzenia tioestru. Dodatkowo stabilizuje cały kompleks pośredni, aż do momentu przyłączenia fosforanu nieorganicznego i wytworzenia finalnego produktu reakcji, czyli 1,3-bisfosfoglicerynianu (Butterfield i in., 2010; Cowan-Jacob i in., 2003; Didierjean i in., 2003). Do innych ważnych reszt aminokwasowych, nieuczestniczących bezpośrednio w katalizowanej reakcji należą: Arg-231 oraz Thr-179. Odpowiadają one za właściwe położenie i wiązanie substratu (G-3-P), a w przypadku Arg-231 także za uwolnienie produktu z centrum aktywnego po zajściu katalizowanej reakcji (Butterfield i in., 2010; Didierjean i in., 2003). Za właściwą lokalizację koenzymu w centrum aktywnym odpowiadają natomiast, między innymi reszty: Tyr-317, Ile-11, Asp-32 i Arg-10 (Butterfield i in., 2010).

Odwracalna przemiana pirogronianu w mleczan katalizowana przez LDH, odbywa się z bezpośrednim udziałem jedynie His-192 oraz koenzymu NADH i jest niezależna od obecności reszt tiolowych cysteiny, które nie są zlokalizowane w obszarze centrum aktywnego enzymu. W czasie tej reakcji, His-192 tworzy wiązanie wodorowe z tlenem grupy karbonylowej kwasu pirogronowego, pełniąc rolę donora protonu w katalizowanej przez NADH reakcji redukcji i utworzenia kwasu mlekowego (Świderek i in., 2009). Pozostałe reszty aminokwasowe takie jak: Arg-105, Arg-168, Thr-247 poprzez liczne wiązania wodorowe, stabilizują położenie pirogronianu w centrum aktywnym i ułatwiają zajście katalizowanej reakcji (Świderek i in., 2009).

Jak wspomniano powyżej, Cys-149 GAPDH dzięki niskiej wartości pKa występuje w formie nukleofilowego anionu tiolowego, co czyni ją wysoce podatną na oksydacyjne modyfikacje. Co więcej, tworzenie międzycząsteczkowych wiązań disiarczkowych z konkretnym udziałem oksydacyjnie zmienionej Cys-149 jest warunkiem koniecznym do agregacji GAPDH w warunkach stresu oksydacyjnego

i nitrozacyjnego (Nakajima i in. 2007, 2009). Oligomeryzacja i agregacja GAPDH jest zwykle poprzedzona utratą czwartorzędowej struktury białka i jego częściowym rozfałdowaniem, co ułatwia tworzenie mostków disiarczkowych między różnymi łańcuchami polipeptydowymi, angażując oprócz Cys-149 także Cys-153, Cys-244 oraz Cys-281 (Muronetz i in., 2017).

Na potrzeby realizacji celów pracy doktorskiej reaktywne formy tlenu i azotu były generowane metodą radiacyjną, enzymatyczną oraz chemiczną. W przypadku metody radiacyjnej, w wyniku radiolizy cząsteczek wody w warunkach tlenowych, otrzymano układ zawierający jednocześnie rodniki: $^{\cdot}OH$, $O_2^{\cdot -}$ oraz H_2O_2. Wydajności radiacyjne G ($\mu mol \cdot J^{-1}$) tworzenia poszczególnych produktów radiolizy wody w badanych warunkach wynoszą: $^{\cdot}OH$ (0,28), $O_2^{\cdot -}$ (0,34), H_2O_2 (0,073), co jest tożsame ze stężeniem otrzymanych RFT wyrażonym w $\mu M \cdot Gy^{-1}$ (Khouri i in. 2004; Le Caër 2011). Układ badawczy zawierający jedynie $O_2^{\cdot -}$ otrzymano w enzymatycznej reakcji przemiany ksantyny w kwas moczowy, katalizowanej przez oksydazę ksantynową w obecności katalazy. Natomiast tlenek azotu (NO), jako przedstawiciel RFA, generowano z użyciem zmodyfikowanej metody zaproponowanej przez Opländer i in. (2010). W metodzie tej, NO powstaje jako produkt fotolitycznego rozkładu azotynu sodu w obecności kwasu askorbinowego.

Ocenę wrażliwości GAPDH i LDH na generowane RFT i RFA, określono poprzez stopień zmian w ich funkcji i strukturze. Zmiany funkcjonalne w badanych białkach oszacowano na podstawie spektrofotometrycznej oceny ich zdolności do redukcji lub utleniania NAD(H), jako kofaktora w katalizowanych przez te enzymy reakcjach. Zmiany strukturalne jako konsekwencja oksydacyjnych modyfikacji lub uszkodzeń, oszacowano z użyciem szeregu metod biofizycznych. Oksydacyjne modyfikacje reszt cysteiny określono spektrofotometryczną metodą Ellmana. Oszacowano także potencjalne miejsca S-nitrozylacji reszt cysteiny badanych białek narzędziami bioinformatycznymi. Zmiany konformacyjne wywołane przez RFT lub RFA, w tym zawartość poszczególnych rodzajów struktury drugorzędowej określono metodą dichroizmu kołowego (CD), a zmiany w ekspozycji obszarów hydrofobowych określono z użyciem znacznika fluorescencyjnego bis-ANS. Wpływ RFT na strukturę czwartorzędową, a także zależną od RFT fragmentację łańcucha polipeptydowego GAPDH i LDH zbadano wykorzystując metodę wysokosprawnej chromatografii cieczowej (HPLC).

Po zbadaniu wpływu RFT i RFA na GAPDH i LDH, kolejnym głównym celem prezentowanych prac, było określenie roli melatoniny oraz resweratrolu, jako związków

mogących zapobiegać oksydacyjnym modyfikacjom i uszkodzeniom badanych enzymów.

Ocenę efektywności wybranych do badań przeciwutleniaczy w zapobieganiu oksydacyjnym modyfikacjom GAPDH i LDH (oba o stężeniu 2μM) zrealizowano, wykonując wspomniane oznaczenia zmian funkcji i struktury enzymów w obecności odpowiednich RFT i RFA oraz 50 lub 100 μM melatoniny i resweratrolu. Dodatkowo w układzie zawierającym jedynie anionorodnik ponadtlenkowy oraz 1,4 μM GAPDH porównano właściwości antyoksydacyjne 50 μM resweratrolu i 50 μM trans-3,3′,5,5′-tetrahydroksy-4′-metoksystilbenu (THMS), strukturalnej pochodnej resweratrolu.

Neuroprotekcyjne działanie zarówno melatoniny jak i resweratrolu oraz jego polifenolowych pochodnych, opiera się przede wszystkim na właściwościach neutralizujących RFT i RFA, co znacznie redukuje poziom stresu oksydacyjnego i oksydacyjne uszkodzenia makrocząsteczek. Jednak związki te, a w szczególności polifenole oraz ich produkty utlenienia, mogą wywierać istotny wpływ na funkcję i strukturę białek (Brudzynski i Maldonado-Alvarez, 2015; Kirsch i Groot, 2008; Sęczyk i in., 2019). Wiele badań wskazuje także, iż możliwość występowania niekowalencyjnych oddziaływań między badanymi związkami lub ich pochodnymi a białkami, może być istotna z punktu widzenia ich działania antyagregacyjnego (Andarzi Gargari i in., 2018; Balmik i in., 2020; Ladiwala i in., 2010). Dlatego również w tej pracy, podjęto próbę określenia możliwości interakcji melatoniny i resweratrolu z cząsteczkami GAPDH i LDH wykorzystując w tym celu metody modelowania molekularnego. W przypadku GAPDH, resweratrolu i THMS użyto także metod biofizycznych. Oceniono gaszenie fluorescencji i czas życia fluorescencji tryptofanów oraz wartość potencjału ζ GAPDH w obecności potencjalnych ligandów, w tym resweratrolu i THMS.

5 Dyskusja najważniejszych wyników

5.1 Wpływu RFT i RFA na funkcję i strukturę GAPDH i LDH

Uzyskane we wszystkich trzech publikacjach (1-3) składających się na niniejszą rozprawę doktorską wyniki potwierdzają, iż GAPDH jest szczególnie wrażliwa na oksydacyjne modyfikacje na tle innych białek o podobnej strukturze, takich jak LDH. Umiarkowany stres oksydacyjny lub nitrozacyjny jest przyczyną odwracalnych modyfikacji reszt aminokwasów w białkach (Cai i Yan, 2013). Takie warunki w pracy

(2) uzyskano poddając GAPDH działaniu tlenku azotu. Przy zastosowanym w badaniach maksymalnym stężeniu NO wynoszącym 170 μM, uzyskano spadek aktywności GAPDH o około 20%, w tych samych warunkach LDH zachowało pełną aktywność (2, Fig. 1). Spadek zawartości wolnych reszt tiolowych oraz przywrócenie pełnej aktywności GAPDH po potraktowaniu enzymu zredukowanym glutationem oraz ditiotreitolem (DTT), świadczyło o odwracalnej modyfikacji reszt Cys (2, Fig.2, Fig.3). Brak zmian aktywności LDH jako enzymu, którego aktywność nie zależy od obecności reszt Cys w centrum aktywnym, jedynie potwierdziło możliwość S-nitrozylacji Cys-149 GAPDH.

Nawet w warunkach umiarkowanego stresu nitrozacyjnego, nie wszystkie cysteiny ulegają w równym stopniu modyfikacji z udziałem NO (Fernando i in., 2019; Nakamura i in., 2015). Wśród najważniejszych czynników strukturalnych danego białka, które zwiększają prawdopodobieństwo modyfikacji określonej cysteiny, należy wymienić: niską wartość pK_a reszty tiolowej cysteiny, obecność w sąsiedztwie reszt cysteiny reszt aminokwasów zasadowych i kwasowych (His, Arg, Lys, Asp, Glu) oraz brak reszt aminokwasów o cząsteczkach na tyle dużych, aby stanowiły zawadę przestrzenną dla tej modyfikacji (Phe, Tyr, Leu). Duże znaczenie ma także stężenie samego tlenku azotu oraz obecność w strukturze trzeciorzędowej białka hydrofobowych nisz, które stanowiłyby rezerwuar dla tego czynnika modyfikującego (Cheng i in., 2014; Fernando i in., 2019). Biorąc pod uwagę część, z powyższych czynników określających specyficzność S-nitrozylacji, dokonano predykcji potencjalnych miejsc dla tej modyfikacji w obu badanych białkach z użyciem programu GPS-SNO 1.0. Określono, iż GAPDH posiada trzy potencjalne miejsca S-nitrozylacji, w tym na resztach Cys-149, Cys-153 oraz Cys-244, w przypadku LDH jedynie reszta Cys-162 jest potencjalnie dostępna dla tej modyfikacji (2, Fig.8).

Znacznie większy poziom oksydacyjnych uszkodzeń GAPDH i LDH zaobserwowano w warunkach, w których RFT były generowane w wyniku napromieniowania roztworów białek promieniowaniem X. Przy użytych w pracy (1) dawkach promieniowania 63 i 420 Gy obserwowano całkowitą inaktywację odpowiednio GAPDH i LDH (1, Fig. 2). Powstający w tych warunkach między innymi rodnik $^{\cdot}OH$, ma silne właściwości utleniające oraz charakteryzuje się wysoką reaktywnością (stałe szybkości reakcji z badanymi białkami limitowane są dyfuzją i wynoszą $\sim 2 \cdot 10^{11}\ M^{-1}s^{-1}$) oraz niską selektywnością (1, Tab. 4). Na podstawie oceny wydajności radiacyjnej inaktywacji (G) określono, iż GAPDH ($G = 7{,}28 \cdot 10^{-2}\ \mu mol \cdot J^{-1}$) jest ~6,7 razy bardziej wrażliwa na radiacyjną inaktywację w porównaniu do LDH ($G = 1{,}09 \cdot 10^{-2}\ \mu mol \cdot J^{-1}$)

(1, Tab. 1, Fig. 2). W odróżnieniu od S-nitrozylacji, inaktywacja GAPDH z udziałem RFT generowanych nawet przy najniższych użytych w pracy dawkach promieniowania, nie była całkowicie odwracalna przy użyciu DTT (1, Fig. 3). To sugeruje oprócz modyfikacji innych reszt aminokwasowych mających pośredni wpływ na aktywność i strukturę enzymu, także nieodwracalne modyfikacje reszt Cys, takie jak S-sulfinacja czy S-sulfonacja. W przeciwieństwie do inaktywowanego GAPDH, aktywność napromieniowanego LDH była całkowicie niewrażliwa na obecność czynników redukujących (1, Fig. 3). Taka obserwacja, potwierdza, że aktywność tego enzymu nie zależy od utleniania reszt cysteiny, co wynika z budowy jego centrum aktywnego. Jednocześnie mniejsza wrażliwość na radiacyjną inaktywację sugeruje, iż uszkodzenia reszt His-192 czy reszt argininy centrum aktywnego LDH, zachodzą przy znacznie większym poziomie oksydacyjnych uszkodzeń struktury białka. To założenie potwierdzają wyniki uzyskane metodą dichroizmu kołowego. Podczas gdy całkowitej inaktywacji GAPDH (dawka 210 Gy) nie towarzyszyły zmiany w strukturze drugorzędowej, to prawie całkowita inaktywacja LDH (dawka 420 Gy) wiązała się z istotnymi zmianami strukturalnymi enzymu, w tym ~7% wzrostem zawartości struktury β i ~9% spadkiem zawartości struktury α-helikalnej (1, Tab. 2, Fig. 5). Obserwowane różnice w poziomie oksydacyjnych uszkodzeń obu enzymów, mogą także wynikać z mniejszej wrażliwości LDH na działanie generowanego radiacyjnie $O_2^{\cdot -}$. Świadczą o tym niższe wartości stałej szybkości reakcji $O_2^{\cdot -}$ z LDH (10^5 $M^{-1} \cdot s^{-1}$) w porównaniu do szybkości reakcji tego rodnika z GAPDH ($2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$) (1, Tab. 4).

Oprócz większej wrażliwości na radiacyjną inaktywację, wyniki uzyskane w trakcie rozdziałów chromatograficznych napromieniowanych białek potwierdziły także większą wrażliwość GAPDH na indukowaną działaniem RFT utratę struktury czwartorzędowej (1, Fig. 6, 7). Porównanie średnich wydajności radiacyjnej destrukcji (G) struktury tetramerycznej enzymów wykazało, że GAPDH ($G = 7{,}36 \cdot 10^{-3}$ $\mu mol \cdot J^{-1}$) jest ~1,6 razy bardziej wrażliwa na radiacyjną utratę struktury czwartorzędowej, czy fragmentację łańcucha polipeptydowego w porównaniu do LDH ($G = 4{,}69 \cdot 10^{-3}$ $\mu mol \cdot J^{-1}$) (1, Tab. 3). Zmiany konformacyjne w strukturze GAPDH, w tym utrata struktury czwartorzędowej w warunkach stresu oksydacyjnego, uważana jest za zjawisko bezpośrednio poprzedzające oligomeryzację i agregację tego białka (Muronetz i in., 2017).

Warto zauważyć, iż obserwowana mniejsza różnica między badanymi enzymami dotycząca wydajności radiacyjnej destrukcji struktury tetramerycznej (2-krotna),

w porównaniu do wydajności radiacyjnej inaktywacji (7-krotna), jedynie potwierdza wysoką redoks reaktywność Cys-149 centrum aktywnego GAPDH. Ma tutaj swoje uzasadnienie reguła mówiąca, iż istotniejsze od całkowitego poziomu oksydacyjnych uszkodzeń białka są uszkodzenia (modyfikacje) krytycznych dla pełnionej funkcji czy właściwej struktury reszt aminokwasowych (Davies, 2016).

5.2 Rola melatoniny i resweratrolu w indukowanych przez RFT i RFA zmianach funkcji i struktury GAPDH i LDH

5.2.1 Badanie oddziaływań przeciwutleniacz-białko

W celu określenia potencjału melatoniny i resweratrolu do zapobiegania oksydacyjnym modyfikacjom i uszkodzeniom białek, zbadano możliwość interakcji obu związków z cząsteczkami GAPDH i LDH. Metodą modelowania molekularnego, określono, iż GAPDH może na swojej powierzchni z równym prawdopodobieństwem związać do 10 cząsteczek resweratrolu i 18 cząsteczek melatoniny, w przypadku LDH jest to odpowiednio 27 i 40 cząsteczek (1, 3). Ze względu, iż oba związki wykazują charakter hydrofobowy można się spodziewać, iż ich oddziaływanie z cząsteczkami białka zmniejszy dostęp do jego powierzchni hydrofobowych znaczników fluorescencyjnych, takich jak bis-ANS. W pracy (2, Tab. 2) zaobserwowano znacznie większy spadek emisji fluorescencji bis-ANS po związaniu z białkiem w obecności obu przeciwutleniaczy w przypadku LDH, w porównaniu z GAPDH. Badanie to, potwierdza przewidywania wiązania większej liczby cząsteczek zarówno resweratrolu jak i melatoniny na powierzchni LDH.

Bardziej szczegółowe badania interakcji przeciwutleniacz-białko wykonano z udziałem GAPDH i resweratrolu. Dokowanie molekularne ligandu w centrum aktywnym enzymu, pozwoliło określić, iż między resweratrolem a resztami aminokwasów centrum aktywnego GAPDH występują oddziaływania niekowalencyjne, w tym wiązania wodorowe (Arg-231, Ser-119) oraz oddziaływania hydrofobowe (His-176, Tyr-317) (3, Tab. 2, Fig.2). Jak wspomniano wcześniej, zarówno His-176, Arg-231 oraz Tyr-317 odgrywają istotną rolę w centrum aktywnym GAPDH, dlatego oddziaływania te są prawdopodobną przyczyną częściowej (~5%) inaktywacji GAPDH w obecności 50 μM resweratrolu (3, Fig.4).

Możliwość występowania oddziaływań polifenol-białko, zweryfikowano następnie metodami biofizycznymi, w tym metodą gaszenia fluorescencji. W pracy założono, iż przy zastosowanej długości fali wzbudzenia 280 nm fluorescencja białka pochodzi głównie od reszt tryptofanu, których GAPDH posiada trzy na każdy monomer, w tym Trp-84, Trp-193 oraz Trp-310. Miareczkowanie roztworu białka rosnącymi stężeniami resweratrolu powodowało gaszenie fluorescencji GAPDH, a nieliniowy charakter krzywej Sterna-Volmera sugerował obecność zarówno mechanizmu statycznego jak i dynamicznego (3, Fig. 5A, 5B). Uważa się, iż nieliniowy kształt krzywej Sterna-Volmera i obecność dwóch stałych gaszenia (K_{SV1} i K_{SV2}), może być także związany z niejednakowym dostępem wygaszacza do fluoroforów, co dodatkowo potwierdzają dwie składowe (τ_1 i τ_2) czasu życia fluorescencji tryptofanów (3, Fig. 5B, Tab. 3, Tab. 4). W przypadku GAPDH w pierwszej kolejności gaszeniu ulegają reszty Trp-193 i Trp-84, położone odpowiednio na powierzchni cząsteczki białka i w jej bezpośrednim sąsiedztwie. Jest to zbieżne z wynikami modelowania molekularnego, które potwierdziły obecność Trp-193 w bliskim sąsiedztwie wszystkich 11 potencjalnych miejsc wiązania resweratrolu oraz Trp-84 dla którego liczba ta wynosiła 8 miejsc wiązania.

W zakresie niskich stężeń resweratrolu proces gaszenia fluorescencji miał charakter gaszenia statycznego, co sugeruje powstanie kompleksu między białkiem a wygaszaczem. Statyczny, a nie dynamiczny charakter gaszenia fluorescencji, potwierdzają praktycznie niezmienne wartości czasu życia fluorescencji (τ_1) najbardziej dostępnych dla wygaszacza reszt Trp GAPDH wynoszące 1,35 ± 0,02 ns bez obecności wygaszacza oraz 1,36 ± 0,02 ns w obecności 5 μM resweratrolu (3, Tab. 4). Otrzymane wyniki gaszenia fluorescencji korelują z obserwowanymi największymi zmianami ładunku i tym samym potencjału ζ na powierzchni cząsteczki GAPDH z ok. (-5) mV bez przeciwutleniacza do ok. (-9) mV przy stężeniu polifenolu wynoszącym poniżej 5 μM (3, Fig. 6). Co więcej, przesunięcie maksimum widma emisji fluorescencji GAPDH z ~339 nm w stronę fal dłuższych do 344 nm wraz z rosnącym stężeniem resweratrolu, sugeruje zmiany konformacyjne GAPDH i ekspozycję głębiej położonych fluoroforów, będące skutkiem oddziaływań białko-wygaszacz (3, Fig. 5A) (Lu i in., 2016; Sun i in., 2017).

Resweratrol jako polifenol przyjmowany z dietą charakteryzuje się dosyć niską biodostępnością, dlatego wciąż trwają poszukiwania naturalnych związków o podobnych

właściwościach antyoksydacyjnych, lecz lepszych właściwościach farmakokinetycznych oraz farmakologicznych (Aldawsari i Velázquez-Martínez, 2015; Chan i in., 2019).

Użyta w pracy (3) pochodna resweratrolu trans-3,3′,5,5′-tetrahydroksy-4′-metoksystilben (THMS), dzięki obecności grupy -OCH_3 w pozycji 4' pierścienia B stilbenu, wykazuje lepsze właściwości lipofilowe, w tym możliwość lepszego przenikania przez błony biologiczne w porównaniu z resweratrolem (Chan i in., 2019). Wyniki badań zawarte w pracy (3) wskazują, iż w porównaniu z resweratrolem pochodna ta, dzięki obecności także większej liczby reszt -OH (dodatkowo w pozycjach 3', 5' pierścienia B stilbenu), tworzy znacznie więcej wiązań wodorowych z resztami aminokwasów centrum aktywnego GAPDH i silniej inaktywuje enzym (3, Tab. 2, Fig. 4). Równocześnie ze względu na znacznie większe rozmiary cząsteczki jedynie 8 cząsteczek THMS może zostać związanych na powierzchni GAPDH (3, Tab. 1 i 2, Fig. 2B). Z tego względu, THMS słabiej gasi fluorescencję Trp, nie zmienia położenia maksimum fluorescencji Trp, a także w mniejszym stopniu wpływa na zmiany potencjału ζ GAPDH (3, Fig. 5B, Fig. 6). Pochodne metoksylowe resweratrolu mimo obiecujących właściwości antyagregacyjnych, charakteryzują się znikomymi właściwościami antyoksydacyjnymi, szczególnie te podstawione w pozycji 4' pierścienia B stilbenu, jak to ma miejsce w przypadku THMS (Derf i in., 2018). Uzyskane w pracy (3) wyniki potwierdzają te założenia w odniesieniu do anionorodnika ponadtlenkowego. W pracy tej nie zaobserwowano istotnych różnic w poziomie inaktywacji GAPDH w obecności $O_2^{\cdot -}$ i THMS w porównaniu do samego $O_2^{\cdot -}$ (3, Fig. 7).

5.2.2 Prooksydacyjne właściwości melatoniny i resweratrolu

Zarówno resweratrol jak i melatonina są uważane za dobre przeciwutleniacze, które efektywnie neutralizują szereg RFT i RFA, o czym świadczą wysokie wartości stałych szybkości tych reakcji (1, Tab. 4, Galano i Reiter, 2018; Mahal i Mukherjee, 2006). Wyjątek stanowi melatonina, która ze względu na niską wartość stałej szybkości reakcji z anionorodnikiem ponadtlenkowym (~10^4 M^{-1} s^{-1}), nie jest uważana za dobry zmiatacz tego rodnika zarówno *in vitro* jak i *in vivo* (Galano i in., 2011). Neutralizacja RFT i RFA, w tym NO, NO_2, $O_2^{\cdot -}$ oraz $^{\cdot}OH$ z udziałem resweratrolu i melatoniny odbywa się poprzez szereg mechanizmów. Należą do nich między innymi mechanizm przeniesienia elektronu SET (ang. single electron transfer) lub atomu wodoru HAT (ang. hydrogen atom transfer) z przeciwutleniacza na rodnik lub bezpośrednie przyłączenie

rodnika do cząsteczki przeciwutleniacza określane jako RAF (ang. radical adduct formation) (Galano i Reiter 2018; Iuga 2012). Należy pamiętać o tym, iż reakcje rodnikowe zwykle nie zachodzą ściśle według jednego mechanizmu, dając przy tym różne strukturalnie pierwotne rodniki przeciwutleniaczy. Rodniki te, podobnie jak same RFT i RFA mogą mieć wpływ na funkcję i strukturę białek.

W pracy (2 i 3) wykazano, iż powstające w czasie reakcji z RFT i RFA produkty utlenienia melatoniny i resweratrolu wykazują działanie prooksydacyjne, przyczyniając się do zwiększenia oksydacyjnych uszkodzeń GAPDH. W pracy (2) powstająca N1-nitrozomelatonina, jako końcowy produkt reakcji melatoniny z NO w obecności O_2, była odpowiedzialna za ~26% wzrost inaktywacji GAPDH, w porównaniu z enzymem poddanym działaniu jedynie RFA (2, Fig. 1, 4). Inaktywacji tej towarzyszył także ~12% spadek zawartości wolnych reszt tiolowych i ~14% zmniejszenie dostępnych dla znacznika bis-ANS obszarów hydrofobowych na powierzchni GAPDH, w porównaniu z białkiem poddanym działaniu jedynie NO (2, Fig. 2, 5, Tab. 2). Biorąc pod uwagę fakt, iż GAPDH może na swojej powierzchni związać do 18 cząsteczek melatoniny, w tych warunkach N1-nitrozomelatonina stanowiła rezerwuar i donor NO, który potęgował S-nitrozylację reszt Cys białka. Zwiększony poziom modyfikacji GAPDH, oprócz wpływu na jej aktywność czy funkcje niezwiązane z glikolizą, może być przyczyną zmian konformacyjnych białka prowadzących do jego agregacji (Kubo i in., 2016; Nakajima i in., 2007, 2009).

Podobny wpływ na funkcję i strukturę GAPDH w badanych warunkach miały powstające w reakcji z NO i NO_2 rodniki resweratrolu. W większości reakcji rodnikowych z udziałem tego przeciwutleniacza oderwanie atomu wodoru lub elektronu odbywa się preferencyjnie z reszty 4'-OH pierścienia B stilbenu. Towarzyszy temu powstanie rodnika fenoksylowego resweratrolu (RSV-O˙). Powstający rodnik przyjmuje strukturę semichinonu, w której niesparowany elektron jest stabilizowany przez rezonans obu pierścieni aromatycznych (A i B) stilbenu (Iuga i in., 2012). W badaniach z pochodnymi resweratrolu Lu i in. (2017) określili, iż w reakcji z NO przeważa mechanizm przyłączenia tego rodnika do węgla C7 podwójnego wiązania układu stilbenu oraz w mniejszym stopniu oderwanie atomu wodoru od reszty 4'-OH z utworzeniem rodnika fenoksylowego. W pracy (2) zaobserwowano, iż resweratrol w obecności NO (170 μM) nasilał inaktywację GAPDH o kolejne ~30% oraz powodował większe zmiany konformacyjne, wyrażone 36% zmniejszeniem fluorescencji bis-ANS w porównaniu do GAPDH poddanego działaniu jedynie NO (2, Fig. 4, 6, Tab. 2). Pomimo braku

weryfikacji eksperymentalnej, można przypuszczać, iż powstające w reakcji z NO rodniki resweratrolu są przyczyną powstania między innymi rodników tiolowych, w tym Cys-149 centrum aktywnego. Rodniki te ulegając dalszym reakcjom utlenienia, prowadzą do inaktywacji enzymu. Inną prawdopodobną przyczyną efektu prooksydacyjnego resweratrolu w badanych warunkach jest kowalencyjna modyfikacja reszt cysteiny z udziałem rodnika polifenolu. Zaproponowany mechanizm jest także zgodny z wynikami uzyskanymi w pracy 3. W pracy tej, powstające w reakcji z anionorodnikiem ponadtlenkowym rodniki resweratrolu, inaktywowały GAPDH w większym stopniu niż sam anionorodnik (3, Fig. 7).

W badaniach z udziałem tlenku azotu, resweratrolu i melatoniny aktywność LDH nie uległa istotnym zmianom zarówno pod wpływem działania N1-nitrozomelatoniny, jak i rodników resweratrolu, podobnie zmianie nie uległa zawartość wolnych reszt tiolowych w LDH (2, Fig. 4, 5, 6, 7). Mniejszy był również poziom zmian konformacyjnych białka. Obniżenie fluorescencji bis-ANS zarówno w obecności resweratrolu jak i melatoniny oraz NO, było o ~30% niższe w porównaniu do wartości fluorescencji znacznika w obecności NO bez przeciwutleniaczy (2, Tab. 2). Jednym z powodów braku wrażliwości jest z pewnością fakt, iż aktywność tego enzymu nie zależy od obecności cystein w centrum aktywnym, które są podstawowym celem związków S-nitrozylujących, takich jak nitrozomelatonina. Co więcej, LDH może związać na swojej powierzchni prawie trzy razy więcej cząsteczek resweratrolu niż GAPDH, można zatem uznać, iż jest także dużo mniej strukturalnie i funkcjonalnie wrażliwa na działanie powstających w reakcji z NO rodników resweratrolu.

5.2.3 Antyoksydacyjne właściwości melatoniny i resweratrolu

Ochronny wpływ na funkcję i strukturę GAPDH i LDH obu użytych przeciwutleniaczy zaobserwowano w układzie zawierającym RFT generowane radiacyjnie (1). Resweratrol zmniejszał poziom radiacyjnej inaktywacji GAPDH ~2-krotnie ($G = 3{,}16 \cdot 10^{-2}$ $\mu mol \cdot J^{-1}$), a LDH ~5,5-krotnie ($G = 0{,}2 \cdot 10^{-2}$ $\mu mol \cdot J^{-1}$) w porównaniu do enzymów naświetlanych bez resweratrolu (1, Tab.1, Fig. 2A, 2B). Melatonina wykazywała znacznie niższy poziom ochrony przed inaktywacją w przypadku GAPDH. Zaobserwowano brak istotnych zmian w poziomie inaktywacji GAPDH naświetlanej w obecności melatoniny ($G = 5{,}67 \cdot 10^{-2}$ $\mu mol \cdot J^{-1}$), natomiast w przypadku LDH melatonina zmniejszała poziom inaktywacji enzymu ~4-krotnie

(G = 0.29 $\cdot 10^{-2}$ μmol·J^{-1}) w porównaniu do enzymów naświetlanych bez przeciwutleniacza (1, Tab. 1, Fig. 2A, 2B). Jak łatwo zauważyć, oba związki w większym stopniu chroniły przed zmianami funkcjonalnymi LDH niż GAPDH. W przypadku GAPDH efekt ochronny był związany w większym stopniu z ochroną przed utlenieniem reszt Cys, które są niezbędne do aktywności enzymu. Przy użytej dawce promieniowania wynoszącej 105 Gy resweratrol zmniejszał poziom utlenienia grup tiolowych GAPDH o ~30% w porównaniu do enzymu naświetlanego bez przeciwutleniacza, podczas gdy melatonina w tych warunkach obniżała poziom utlenienia grup -SH o ~20% (1, Fig. 4A). Oba przeciwutleniacze w podobnym stopniu chroniły GAPDH przed zmianami struktury czwartorzędowej. Wydajności radiacyjnej utraty struktury tetramerycznej G wynosiły $2{,}71 \cdot 10^{-3}$ i $1{,}81 \cdot 10^{-3}$ μmol·J^{-1} odpowiednio dla GAPDH naświetlanego w obecności resweratrolu i melatoniny (1, Tab. 3, Fig. 6A). W odniesieniu do LDH większy poziom ochrony przed inaktywacją był konsekwencją zapobiegania zmianom w strukturze drugo- i czwartorzędowej enzymu. Melatonina zmniejszała indukowane promieniowaniem X (420 Gy) zmiany w zawartości struktury β w LDH o ~3% oraz α-helisy o ~4% w porównaniu do białka napromieniowanego bez przeciwutleniacza (1, Tab. 2, Fig. 5B). W tych samych warunkach, resweratrol niemal całkowicie zapobiegał zmianom w zawartości poszczególnych struktur drugorzędowych enzymu (1, Tab. 2). Analiza wydajności radiacyjnej redukcji frakcji tetramerycznej białka wykazała, iż resweratrol zmniejszał zależną od promieniowania utratę czwartorzędowej struktury LDH nawet 13-krotnie (G = $0.35 \cdot 10^{-3}$ μmol·J^{-1}), podczas gdy melatonina podobnie jak w przypadku GAPDH jedynie ~4-krotnie (G = $1{,}33 \cdot 10^{-3}$ μmol·J^{-1}) (1, Tab. 3).

Jest kilka przyczyn, z których wynikają różnice w poziomie ochrony GAPDH i LDH przez badane przeciwutleniacze. W warunkach, w których RFT generowane są radiacyjnie, inicjatorem utlenienia zarówno resweratrolu jak i melatoniny jest rodnik ˙OH (Bonnefont-Rousselot i in. 2011; Camont i in. 2012). Jak wspomniano wcześniej, rodnik wodorotlenowy jest mało specyficzny, więc także w reakcji z melatoniną jak i resweratrolem daje szereg różnych strukturalnie pierwotnych rodników (Bonnefont-Rousselot i in. 2011; Camont i in. 2012; Galano i Reiter, 2018). Podobnie jak w pracy (2) tak i w tym przypadku LDH okazało się dużo mniej wrażliwe zarówno na działanie reaktywnych form, jak i samych rodników przeciwutleniaczy. Z drugiej strony, większa liczba cząsteczek przeciwutleniaczy związana na powierzchni LDH (40 cząsteczek melatoniny i 27 resweratrolu), stanowiła lepszą tarczę, chroniącą białko przed uszkadzającym działaniem rodników ˙OH czy $O_2^{\cdot -}$ w porównaniu do GAPDH, która na

swojej powierzchni wiąże mniej przeciwutleniaczy, odpowiednio 18 cząsteczek melatoniny i 10 resweratrolu. Można wnioskować, iż w warunkach, w których powstają wysoce reaktywne i mało specyficzne formy tlenu, efekt ochronny resweratrolu i melatoniny przeważa nad uszkadzającym działaniem powstających rodników tych związków. Co więcej, biorąc pod uwagę, iż zarówno GAPDH jak i LDH wiążą znacznie więcej cząsteczek melatoniny niż resweratrolu, można przypuszczać, iż powstające w reakcji z ˙OH rodniki melatoniny wykazują znacznie większy efekt uszkadzający niż rodniki resweratrolu. Prawdopodobnie też, silniej oddziałują z resztami aminokwasowymi centrum aktywnego GAPDH, gdyż praktycznie nie chroniły tego enzymu przed radiacyjną inaktywacją.

6 Wnioski i podsumowanie

1. Uzyskane wyniki potwierdzają dużo większą wrażliwość GAPDH na indukowane przez RFT i RFA zmiany funkcjonalne i strukturalne na tle innych dehydrogenaz, takich jak LDH. Wynikają one z różnic strukturalnych, w tym różnic w budowie centrum aktywnego obu enzymów.
2. Metodą modelowania molekularnego oraz metodami biofizycznymi potwierdzono, iż między resweratrolem, melatoniną a cząsteczkami GAPDH i LDH zachodzą oddziaływania niekowalencyjne.
3. W czasie reakcji RFT i RFA z badanymi w pracy antyoksydantami (resweratrol, melatonina) powstają rodniki lub produkty utlenienia resweratrolu i melatoniny, które wywierają istotny wpływ na funkcję i strukturę GAPDH oraz w dużo mniejszym stopniu LDH.
4. Konsekwencją oddziaływania rodników resweratrolu z cząsteczką GAPDH jest wystąpienie efektu prooksydacyjnego, czyli nasilenia inaktywacji lub zmian strukturalnych białka w układach, gdzie generowany był anionorodnik ponadtlenkowy lub tlenek azotu.
5. Podobne prooksydacyjne działanie produktów utlenienia melatoniny w odniesieniu do funkcji i struktury GAPDH zaobserwowano w układzie zawierającym tlenek azotu.
6. Wyraźny efekt antyoksydacyjny badanych związków był obserwowany jedynie w warunkach, w których RFT generowane były radiacyjnie. W tym układzie resweratrol zapobiegał zmianom funkcjonalnym i strukturalnym GAPDH i LDH.

Melatonina natomiast, chroniła przed zmianami strukturalnymi zarówno GAPDH i LDH oraz przed zmianami funkcjonalnymi w istotnym stopniu jedynie cząsteczkę LDH.

7. Oba badane przeciwutleniacze w większym stopniu chroniły przed radiacyjnymi uszkodzeniami LDH w porównaniu z GAPDH. Jest to związane z jednej strony z mniejszą wrażliwością LDH na działanie RFT, a z drugiej na powstające pod wpływem promieniowania rodniki melatoniny i resweratrolu.

Przeprowadzone badania mimo, iż dotyczą prostych układów *in vitro*, dostarczyły cennych informacji na temat interakcji resweratrolu i melatoniny oraz ich produktów utlenienia z badanymi białkami. Jak wynika z przeprowadzonych badań, oba przeciwutleniacze mogą wywierać zarówno efekt pro- jak i antyoksydacyjny, który jest ściśle związany z obecnością określonego rodzaju RFT lub RFA w układzie badawczym.

Wyraźny efekt ochronny przed oksydacyjnymi uszkodzeniami GAPDH i LDH obserwowany w układzie zawierającym generowane radiacyjnie: $^{\cdot}OH$, $O_2^{\cdot -}$, H_2O_2, z pewnością zachęca do dalszych badań, dotyczących wykorzystania tych związków w zapobieganiu indukowanej przez RFT agregacji białek. Badania przeprowadzone w naszym laboratorium, a niebędące częścią niniejszej pracy, jasno wskazują, iż oba przeciwutleniacze zapobiegają indukowanej radiacyjnie agregacji GAPDH.

Podsumowując, wykorzystanie badanych przeciwutleniaczy jako potencjalnego środka zapobiegającego rozwojowi lub progresji, związanych z wiekiem chorób neurodegeneracyjnych wymaga wielu dalszych badań.

7 Streszczenie

Stres oksydacyjny i związane z nim oksydacyjne uszkodzenia makrocząsteczek, w tym białek są przyczyną wielu schorzeń związanych z wiekiem. Dehydrogenaza aldehydu 3-fosfoglicerynowego (GAPDH) jest wielofunkcyjnym enzymem glikolitycznym, który uczestniczy między innymi w przekazywaniu sygnału redoks w komórce. Oddziaływanie oksydacyjnie zmienionej GAPDH z niektórymi białkami, podobnie jak jej indukowana oksydacyjnymi uszkodzeniami agregacja, mogą stanowić istotny czynnik inicjujący śmierć komórkową. Wymienione cechy dotyczą w dużym stopniu komórek nerwowych i powodują, iż GAPDH już od wielu lat zajmuje istotne miejsce w badaniach nad patomechanizmem chorób neurodegeneracyjnych, takich jak

choroba Alzheimera, Parkinsona czy Huntingtona. Aktualnie jedną ze strategii dotyczących profilaktyki tych schorzeń jest zapobieganie niepożądanym, oksydacyjnym modyfikacjom i uszkodzeniom białek, w tym także GAPDH.

W prezentowanej pracy doktorskiej porównano wrażliwość GAPDH i dehydrogenazy mleczanowej (LDH) na działanie wybranych reaktywnych form tlenu i azotu (RFT i RFA). Następnie oceniono skuteczność dwóch naturalnie występujących przeciwutleniaczy: melatoniny i resweratrolu w przeciwdziałaniu indukowanym stresem oksydacyjnym zmianom funkcji i struktury badanych dehydrogenaz. W celu realizacji założeń pracy użyto metod modelowania molekularnego, metod spektroskopii: UV-VIS, dichroizmu kołowego, fluorescencyjnej oraz metody wysokosprawnej chromatografii cieczowej (HPLC).

Uzyskane wyniki potwierdziły znacznie większy wpływ RFT i RFA na funkcję i strukturę GAPDH w porównaniu z LDH. Jest to związane z różnicą w budowie obu enzymów, a w szczególności ich centrum aktywnego. W centrum aktywnym GAPDH obecna jest wysoce reaktywna Cys-149, która łatwo ulega odwracalnym i nieodwracalnym modyfikacjom z udziałem tlenku azotu jak i RFT. W przeciwieństwie do GAPDH na aktywność LDH nie mają wpływu modyfikacje reszt cysteiny (utlenianie, S-nitrozylacja), gdyż te nie występują w obszarze centrum aktywnego enzymu. Skutkiem tego jest brak wrażliwości LDH na zmiany funkcjonalne pod wpływem tlenku azotu. Natomiast indukowana działaniem RFT inaktywacja LDH jest poprzedzona większymi w porównaniu z GAPDH zmianami w strukturze czwarto- i drugorzędowej enzymu.

W kolejnym etapie badań ustalono, iż wybrane przeciwutleniacze wykazują zarówno właściwości pro- jak i antyoksydacyjne w badanych układach. Oznacza to, iż mogą nasilać lub redukować indukowane przez reaktywne formy zmiany funkcji i struktury GAPDH i LDH. Chcąc wyjaśnić przyczyny tego zjawiska, dokonano oceny możliwości oddziaływania obu przeciwutleniaczy z cząsteczkami badanych białek. Zgodnie z przewidywaniami dokowania molekularnego GAPDH i LDH mogą związać na swojej powierzchni resweratrol (10 cząsteczek ligandu GAPDH i 27 cząsteczek ligandu LDH) oraz melatoninę (18 cząsteczek ligandu GAPDH i 40 cząsteczek ligandu LDH). W związku z tym za efekt prooksydacyjny w stosunku do białka mogą odpowiadać rodnikowe lub nierodnikowe produkty reakcji resweratrolu i melatoniny z RFT lub RFA. Przypuszczenia te zweryfikowano w układzie zawierającym tlenek azotu, gdzie powstające rodniki fenoksylowe resweratrolu oraz N-nitrozomelatonina nasilały utlenianie oraz S-nitrozylację Cys-149 GAPDH, a także zmiany konformacyjne

w GAPDH i LDH. Podobnie rodniki resweratrolu powstające w obecności anionorodnika ponadtlenkowego nasilały inaktywację GAPDH. Właściwości prooksydacyjne badanych przeciwutleniaczy przeważały w układach, gdzie generowane były mniej reaktywne, ale bardziej selektywne RFT i RFA. Efekt antyoksydacyjny zaobserwowano natomiast kiedy RFT generowane były radiacyjnie. W tym przypadku związane na powierzchni białka cząsteczki przeciwutleniaczy tworzyły tarczę ochronną przed uszkadzającym działaniem wysoce reaktywnego rodnika wodorotlenowego. LDH, która może związać na swojej powierzchni więcej cząsteczek obu przeciwutleniaczy była wydajniej chroniona przed radiacyjnie indukowaną utratą funkcji i zmianami strukturalnymi w porównaniu do GAPDH. Porównując oba przeciwutleniacze to resweratrol był skuteczniejszy w ochronie GAPDH przed radiacyjną inaktywacją oraz lepiej chronił strukturę drugorzędową i tetrameryczną LDH. Jedną z przyczyn tej różnicy jest z pewnością to, iż radiacyjnie generowane rodniki resweratrolu powodują mniejsze uszkodzenia w badanych białkach w porównaniu z rodnikami melatoniny. Ponadto, według dostępnych danych literaturowych, melatonina praktycznie nie zmiata anionorodnika ponadtlenkowego, który jest obok rodnika wodorotlenowego istotnym składnikiem produktów radiolizy wody w warunkach tlenowych. Wiadomo również, iż GAPDH jest znacznie bardziej wrażliwa na działanie tej RFT w porównaniu z LDH.

Podsumowując uzyskane w pracy wyniki zarówno resweratrol jak i melatonina mogą nasilać lub przeciwdziałać oksydacyjnym uszkodzeniom w badanych enzymach glikolitycznych. O tym, który efekt przeważa decyduje rodzaj RFT lub RFA w badanym układzie, a także strukturalna i funkcjonalna wrażliwość danego białka na działanie produktów reakcji RFT i RFA z resweratrolem lub melatoniną.

8 Abstract

Oxidative stress and associated with it oxidative damage to macromolecules including proteins are the main causes for age-related diseases. Glyceraldehyde-3-phosphate dehydrogenase is a multifunctional glycolytic enzyme that is also involved in redox signaling. Interactions of the oxidatively modified GAPDH with specific proteins together with its oxidative damage induced aggregation are both significant factors initiating cell death. These features are especially common to neuronal cells making GAPDH through years an important object of the studies elucidating pathomechanism of neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases.

One of the prophylaxis strategies against neurodegeneration is to prevent unwanted oxidative modifications and damage to specific proteins including GAPDH.

In the presented thesis it was compared the vulnerability of GAPDH and lactate dehydrogenase (LDH) to selected reactive oxygen and nitrogen species (ROS and RNS). Next, it was assessed the efficacy of two naturally occurring antioxidants: melatonin and resveratrol to counteract the oxidative stress induced structural and functional changes of the studied dehydrogenases. In order to fulfil objectives of the study several methods were used, including molecular modeling, UV-VIS spectroscopy, circular dichroism (CD), fluorescence spectroscopy, and high-performance liquid chromatography (HPLC).

The obtained results confirmed greater ROS and RNS impact on the function and structure of GAPDH compared to LDH. It is due to the structural differences of both enzymes, in particular, within their active sites. GAPDH active site contains highly reactive Cys-149 that is easily reversibly and irreversibly modified with both nitric oxide and ROS. Contrary to GAPDH, LDH activity is not affected by the modifications of cysteine residues (oxidation, S-nitrosylation) as they are not present in the active site region of the enzyme. As a result, function of LDH is insensitive to the effect of nitric oxide. However, ROS induced inactivation of LDH was preceded with greater alterations in the quaternary and secondary structure of the enzyme when compared to GAPDH.

In the next stage of the research it was revealed that studied antioxidants exhibit both pro- and antioxidant properties in studied systems. This indicates that they either increase or decrease functional and structural changes of GAPDH and LDH induced by reactive species. In order to determine the cause of this phenomenon the possibility of interaction between antioxidants and studied proteins was verified. According to molecular docking predictions GAPDH and LDH potentially bind on its surface resveratrol (GAPDH 10 molecules of the ligand and LDH 27 molecules of the ligand) and melatonin (GAPDH 18 molecules of the ligand and LDH 40 molecules of the ligand). Therefore, the prooxidative effect on the protein may be attributable to the action of radical and nonradical products formed in the reaction of melatonin and resveratrol with ROS and RNS. These assumptions were verified in the system containing nitric oxide where formed phenoxyl radical of resveratrol and N-nitroso-melatonin increased oxidation and S-nitrosylation of GAPDH Cys-149 and conformational changes in GAPDH and LDH. Similar effect was observed with resveratrol radicals formed by superoxide anion radical which increased inactivation of GAPDH. Thus, the prooxidative effect of the studied antioxidants was prevalent in the systems containing less reactive but

more selective ROS and RNS. In contrast, antioxidative effect was observed when ROS were generated by X-ray radiation. In this case molecules of the antioxidant bound on the surface of protein created a protective shield against damaging effect of hydroxyl radical. LDH which is able to bind on its surface more molecules of both antioxidants was more effectively protected against radiation-induced loss of function and structure damage when compared to GAPDH. By comparing both antioxidants, it was resveratrol which was more effective in protection of GAPDH against inactivation and LDH against changes in the secondary and quaternary structure. One of the reasons for this difference is certainly the fact that radiation generated resveratrol radicals cause less damage to the studied proteins when compared to melatonin radicals. Furthermore, according to the available literature data melatonin is almost inefficient for scavenging superoxide anion radical which is together with hydroxyl radical important product of water radiolysis in the presence of dioxygen. It is also known, that GAPDH is far more sensitive to this type of ROS in comparison to LDH.

Summarizing the results of the thesis it can be concluded that both resveratrol and melatonin are able to enhance or prevent the oxidative damage to investigated glycolytic enzymes. The final outcome is strongly dependent upon the type of ROS and RNS present in the studied system and additionally structural and functional sensitivity of a particular protein to the effect of the reaction products of melatonin and resveratrol with ROS and RNS.

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10 Pozostały dorobek naukowy autora

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Komunikaty zjazdowe

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9. Rodacka, A., Gerszon, J., Strumiłło, J., Puchała, M. (2013, wrzesień). *Wpływ resweratrolu na komórki nerwiaka płodowego (Neuro-2a) w warunkach stresu oksydacyjnego.* Prezentacja plakatu przedstawiona na XVI Zjeździe Polskiego Towarzystwa Badań Radiacyjnych, Białowieża.

10. Strumiłło, J., Rodacka, A., Puchała, M. (2013, wrzesień). *Wpływ resweratrolu i jego pochodnych na funkcję i strukturę dehydrogenazy aldehydu 3- fosfoglicerynowego w warunkach stresu oksydacyjnego.* Prezentacja plakatu przedstawiona na XVI Zjeździe Polskiego Towarzystwa Badań Radiacyjnych, Białowieża.
11. Strumiłło, J., Rodacka, A., Puchała, M. (2013, wrzesień). *The influence of resveratrol and its derivatives on the structure and function of glyceraldehyde-3-phosphate dehydrogenase under oxidative stress conditions.* Prezentacja plakatu przedstawiona na 48 Zjeździe Polskiego Towarzystwa Biochemicznego. Od sekwencji kwasów nukleinowych do medycyny molekularnej, Toruń.
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ORIGINAL ARTICLE

Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase

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ABSTRACT

Purpose: This work investigates the effect of resveratrol and melatonin on structural and functional changes of two enzymes, lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), exposed to radiation-induced reactive oxygen species.

Materials and methods: Solutions of dehydrogenases with or without antioxidants (resveratrol or melatonin) were irradiated with X-rays under the atmosphere of air and at room temperature ($21 \pm 2\,^{\circ}C$). In order to determine the protective effect of melatonin and resveratrol in radiation-induced damage to GAPDH and LDH spectroscopy and HPLC methods were used. Furthermore, plausible binding sites of melatonin or resveratrol to the GAPDH or LDH molecule were analysed.

Results and conclusions: Resveratrol shows better protective properties in the inactivation of GAPDH when compared to melatonin. LDH does not contain –SH groups in its active site, and is not inactivated by water radiolysis products other than hydroxyl radicals or the secondary radicals of the studied low-molecular-weight compounds. Resveratrol and melatonin protected the structure of LDH to a greater extent than GAPDH. This difference can be attributed to the fact that LDH potentially binds more resveratrol or melatonin molecules (27 binding sites for resveratrol and 40 for melatonin) than GAPDH (10 binding sites for resveratrol and 18 for melatonin).

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KEYWORDS
GAPDH; LDH; radiation; resveratrol; melatonin

Introduction

Reactive oxygen species (ROS) are present in all aerobic organisms as a consequence of normal aerobic metabolism, especially respiration. However, they are also produced as a result of exposure to certain noxious factors such as infectious agents, xenobiotics, pollution, UV light, ionising radiation, ultrasound and cigarette smoke. Under physiological conditions, ROS are neutralised by the antioxidant system, which consists of enzymes such as superoxide dismutase, catalase, glutathione peroxidases and heme oxygenase-1, and non-enzymatic antioxidants including low-molecular-weight compounds such as glutathione (GSH), some vitamins (vitamins C and E), β-carotene and uric acid. Overproduction of ROS may lead to oxidative stress, ultimately causing irreversible damage to cells and tissues. Oxidative stress has been proven to contribute to many pathological conditions, including neurological disorders, cancer, diabetes, atherosclerosis, ischemia/perfusion and asthma (Valko et al. 2007).

Due to their abundance in cells and high reactivity with free radicals, proteins are considered to be the main targets of products of water radiolysis (Du and Gebicki 2004; Davies 2005, 2012; Dahl et al. 2015). The accumulation of damaged proteins contributes to multiple age-related diseases such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) (Jucker and Walke 2011; Höhn et al. 2014). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in particular, endures significant damage under conditions of oxidative stress (Brandes et al. 2009; Hwang et al. 2009; Pérez et al. 2009).

GAPDH is an enzyme of the glycolytic pathway, which catalyses the reversible conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in a reaction that is accompanied by the reduction of NAD^+ to NADH. In addition to its catalytic function, GAPDH is a classic example of a multifunctional protein. Many studies have shown that this enzyme is involved in various cellular processes such as regulation of transcription, DNA repair, microtubule formation and polymerisation, vesicular transport and nuclear RNA transport, to name a few (Seidler 2013; Sirover 2017). Oxidatively modified GAPDH may initiate apoptotic cell death in neuronal cells. Moreover, numerous studies have reported that GAPDH is one of the main components of extra- and intracellular amyloid lesions characteristic of neurodegenerative disorders such as AD and PD (Hara et al. 2005; Verdier et al. 2005; Wang et al. 2005; Naletova et al. 2008; Butterfield et al. 2010).

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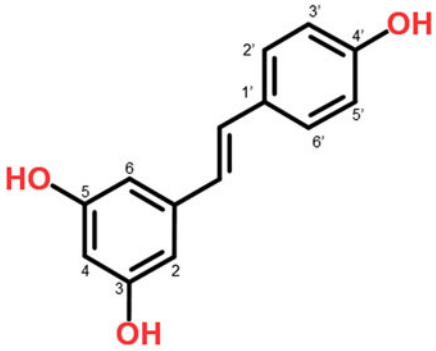
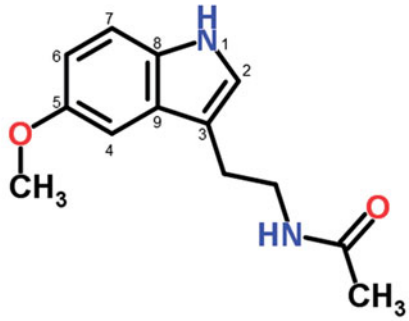


Resveratrol
(3,5,4'-trihydroxy-trans-stilbene)



Melatonin
(N-acetyl-5-methoxytryptamine)

Figure 1. Chemical structures of resveratrol and melatonin.

Protection of proteins against oxidative damage may have a beneficial effect in the early stages of neurodegenerative disease, carcinogenesis and cataracts. Natural polyphenols commonly found in vegetables, fruits, tea and red wine are regarded to possess powerful antioxidative properties (Embuscado 2015; Shahidi and Ambigaipalan 2015; Molino et al. 2016). However, according to several independent studies, antioxidant compounds may also exert pro-oxidative properties under certain conditions. Therefore, it is of great interest to study such properties in simple in vitro systems. Resveratrol (3,5,4′-trihydroxy-trans-stilbene, RSV) and melatonin (N-acetyl-5-methoxytryptamine, MLT) are two widely studied and highly effective antioxidants. Both are good scavengers of •OH radicals, with rate constants (k) of 5×10^9 and 1.2×10^{10} mol^{-1} $dm^3 s^{-1}$ for RSV and MLT, respectively (Mahal et al. 1999; Mahal and Mukherjee 2006). According to Camont et al. (2012), the most probable reactions of •OH radicals with resveratrol are hydrogen abstraction from the hydroxyl group at position C4′, and •OH radical addition to the phenol ring at position C3′ and to the double bond between the rings. In the case of melatonin, the most probable initial products of reactions with •OH radicals are formed as a result of •OH radical addition to the indole ring at positions C2 and C3 and adduct formation with the benzene ring at C7 (Roberts et al. 1998) (Figure 1).

During the reaction of the studied antioxidants (RSV and MLT) with •OH radicals, secondary •RSV and •MLT radicals are formed. These species may protect the protein from •OH-mediated damage, as their efficiency in protein inactivation and destruction is much lower than that of •OH radicals.

The objective of the present work was to compare the efficiency of resveratrol and melatonin in protecting against structural and functional changes of two enzymes, LDH and GAPDH, following X-ray exposure.

Materials and methods

Reagents

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) from rabbit muscle, N-acetyl-5-methoxytryptamine (melatonin, MLT), 3,4′,5-trihydroxy-trans-stilbene (resveratrol, RSV), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), oxidised (NAD) and reduced (NADH) forms of nicotinamide adenine dinucleotide, dithiothreitol (DTT), pyruvic acid and glyceraldehyde-3-phosphate were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of analytical grade, and were purchased from Sigma-Aldrich (St. Louis, MO) or POCH (Gliwice, Poland). All solutions were made with water purified with the Milli-Q system (Millipore Corporation, Billerica, MA).

Preparation of protein and antioxidant solutions

Protein solutions were prepared by dissolving enzymes in 0.02 mol dm^{-3} sodium phosphate buffer at pH 7.4. The enzyme concentrations were determined spectrophotometrically at 280 nm using the extinction coefficient $E^{1\%}$=10 for GAPDH and $E^{1\%}$=14.9 for LDH.

Aqueous solutions of resveratrol were prepared by sonication in 0.02 mol dm^{-3} phosphate buffer (pH 7.4) in ultra-pure water. The concentration was determined spectrophotometrically at 304 nm using a molar extinction coefficient of 30335 mol^{-1} dm^3 cm^{-1}, as reported by Camont et al. (2009). Solutions of resveratrol were kept in the dark to avoid isomerisation into the *cis*-form.

Solutions of melatonin were prepared by directly dissolving the powder in phosphate buffer. The concentration was determined spectrophotometrically at 278 nm using a millimolar extinction coefficient of 6.30 $mmol^{-1}$ dm^3 cm^{-1} (based on the Merck Index).

Irradiation conditions

Solutions of dehydrogenases (2 µmol dm^{-3} in 0.02 mol dm^{-3} sodium phosphate buffer, pH 7.4) with or without antioxidants (resveratrol or melatonin) were irradiated with X-rays under the atmosphere of air and at room temperature (21 ± 2 °C). The concentration of resveratrol or melatonin in the irradiated solutions was 100 µmol dm^{-3}. The dose rate estimated with a modified Fricke dosimeter was 21 Gy/min.

The radiation chemical yield of ferric ions ($G(Fe^{3+})$) used throughout this work was 14.7 mol/100 eV (1.51×10^{-6} mol J^{-1}; German dosimetry protocol, DIN 6800, Teil 31980). Irradiation was performed with a Stabilipan X-ray machine with the following parameters: 195 kV, 18 mA and 1.5-mm aluminium filter. The solution was continuously stirred during irradiation (about 100 rpm).

Enzymatic activity of dehydrogenases

The GAPDH and LDH enzymatic assays were performed as previously described (Rodacka et al. 2010). The enzyme activities were determined based on the rate of reduction of NAD^+ to NADH (for GAPDH) or the rate of oxidation of NADH to NAD^+ (for LDH). Measurements were made at a wavelength of 340 nm. The activity was calculated from the initial slope of the recorded curve. All spectrophotometric measurements were carried out at room temperature (21 ± 2 °C) in a Cary-1 apparatus (Varian, Melbourne, Australia).

The activities of enzymes were determined in two systems: in the first system, the activity was measured 30 min after irradiation of the enzyme solutions; in the second system, enzymatic activity was measured after incubation of the irradiated samples with 1 mM thiol reagent dithiothreitol (DTT) for 30 min.

Sulfhydryl group analysis

Detection of the relative content of –SH groups in the proteins was based on the formation of a coloured product resulting from the reaction of DTNB (Ellman's reagent). Absorbance at 412 nm was measured spectrophotometrically in 4.75 mol dm^{-3} guanidinium chloride. Absorbance measurements were done 15 min after addition of DTNB to the protein solution. Changes in the content of free –SH groups in GAPDH or LDH were calculated as the ratio absorbance of tested samples to absorbance of control sample (not irradiated) × 100.

Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed using a Jasco 815 spectropolarimeter (Jasco, Tokyo, Japan). Far UV measurements were recorded using 0.28 µmol dm^{-3} protein with a 5-mm pathlength cell. Spectra were obtained as the average of three successive scans with a bandwidth of 2.0 nm. The data were expressed as molar residue ellipticities. All data were modified by subtracting the baseline of the respective buffer with or without antioxidant. The secondary structure content of enzymes was calculated using CD spectrum deconvolution software CDNN (Böhm et al. 1992).

High-performance liquid chromatography (HPLC) separations

For GAPDH and LDH preparations, filtration chromatography was performed using the Agilent Zorbax Bio column (GF 250; 250 × 4.6 mm) and a Waters Multisolvent Delivery System (Waters 600E, Waters 717 with an autosampler, Waters 486 absorbance detector). The column was injected with 5 µl of protein solution with a concentration of 2 µmol dm^{-3}, and elution was conducted with 0.2 mol dm^{-3} sodium phosphate buffer (pH 7.4). The eluent flow rate was 0.2 ml/min. The column was calibrated using the following protein standards: thyroglobulin (~670,000 Da, retention time – $t_r = 13.43$ min), α-globulin (~150,000 Da, $t_r = 10.56$ min), ovalbumin (~44,300 Da, $t_r = 11.92$ min) and ribonuclease (~13,434 Da, $t_r = 13.43$ min). For the abovementioned proteins, a linear relationship between the relative retention time and log molecular weight was obtained.

During separation, any resveratrol and melatonin that had not bound to the proteins was adsorbed by the column bed. After separation, the column was flushed with a solution of 40% methanol in 0.05 mol dm^{-3} sodium phosphate buffer (pH 7.4) for approximately 80 min, with a flow rate 0.4 ml/min, to remove the adsorbed antioxidants. The column was then reconditioned with a standard mobile phase (0.2 mol dm^{-3} sodium phosphate buffer, pH 7.4).

Molecular modelling and docking

The 3D structures of resveratrol and melatonin were downloaded from PubChem (CID 445154 and CID 896). Docking studies were conducted using a BIOVIA Discovery Studio (Dassault Systems) molecular simulation system with the LigandFit and CDOCKER algorithms. Molecular graphics were produced using the UCSF Chimera package (Pettersen et al. 2004). The GAPDH and LDH structures used in this work were from the Protein Data Bank, obtained by X-ray diffraction (GAPDH 1J0X and LDH 3H3F). Potential binding sites were derived from cavities in the structure of the receptor based on the algorithm implemented in Discovery Studio. Ligand poses were evaluated and prioritised according to the value of the Dock-Score function (D-S).

Statistical analysis

Results are presented as mean ± standard deviation (SD). The statistical significance of the impact of radiation dose and ligand on protein function and structure was determined using two-way analysis of variance. All analyses were performed using STATISTICA version 13.0 software.

Results

Effects of resveratrol and melatonin on enzyme inactivation by products of water radiolysis

To examine the effect of antioxidants (resveratrol or melatonin) on radiation-induced inactivation of GAPDH and LDH, we exposed dehydrogenases with or without

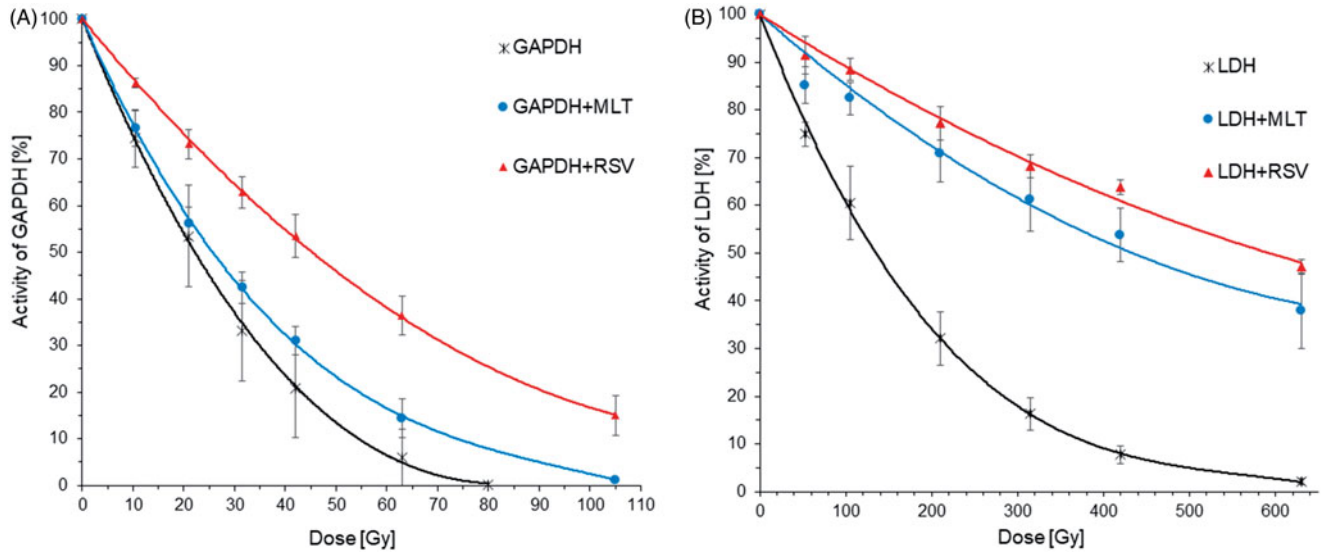


Figure 2. Inactivation of GAPDH (A) and LDH (B) enzymes irradiated in the absence or presence of resveratrol or melatonin. Enzyme activities are expressed as a percentage of the control. Each point represents the mean ± standard deviations of 4–7 independent experiments.

Table 1. Radiolytic yield (*G*) of the inactivation process for GAPDH and LDH.

	GAPDH $G \times 10^2$ (μmol/J)		LDH $G \times 10^2$ (μmol/J)	
Antioxidant	–	+DTT	–	+DTT
–	7.28 ± 0.52	3.23 ± 0.38	1.09 ± 0.06	1.03 ± 0.07
RSV	3.16 ± 0.17	1.00 ± 0.06	0.20 ± 0.01	0.18 ± 0.01
MLT	5.67 ± 0.30	1.85 ± 0.11	0.29 ± 0.01	0.26 ± 0.01

Radiolytic yields were calculated based on the value of the dose D_{37} according to the formula $G = [E]/(\rho \times D_{37})$, where [E] is the enzyme concentration (in mol/dm^3), D_{37} is the irradiation dose (in Gy) at which the enzyme activity was decreased to 37% of the initial activity, and ρ is the density of the solution (≈1 kg/dm^3).

resveratrol or melatonin to X-rays at doses ranging from 0 to 105 Gy for GAPDH and 0 to 630 Gy for LDH. The enzyme activities were measured in two systems: in the first system, the activity was determined 30 min after irradiation; and in the second system, the samples were treated with DTT before measurement of the enzyme activity.

Similarly to our previous work, we showed that GAPDH is about 6.7-times more radiosensitive to inactivation than LDH (Figure 2 and Table 1). Resveratrol was much more efficient in protecting GAPDH from radiation-induced inactivation than melatonin (Table 1). The presence of RSV or melatonin in the irradiated solution protected LDH to a greater extent than GAPDH. Statistical analysis revealed that the changes in GAPDH and LDH activity are dependent on the X-ray dose and ligand presence ($p < .05$). For irradiated GAPDH, *post-hoc* analysis revealed a statistically significant effect of RSV, but not melatonin, on GAPDH activity. For irradiated LDH, a *post-hoc* analysis indicated that both melatonin and RSV have a significant effect on the enzymatic activity of GAPDH and LDH, but their impact is statistically indistinguishable.

Impact of the oxidation of sulfhydryl groups on enzyme inactivation

The incubation of irradiated GAPDH with the thiol reagent DTT significantly (but not completely) restored its catalytic activity (Figure 3). These findings suggest that the inactivation of GAPDH is largely dependent on the creation of sulfenic acid or disulfide bonds, in which Cys 149 participates. In contrast, DTT did not restore the activity of LDH. This means that the activity of this enzyme is not significantly affected by the oxidative modification of cysteine residues.

Oxidation of the –SH groups of cysteine residues in the presence or absence of antioxidants was quantified. Free –SH groups were determined by the Ellman method. Measurements were performed in an environment containing 4.75 mol dm^{-3} guanidine hydrochloride, in which proteins are completely denatured and all –SH groups are available for the DTNB.

Cysteine residues were the most readily oxidised in protein solutions that had been irradiated in the absence of antioxidants. At a radiation dose of 105 Gy, the relative content of free –SH groups was approximately 43% for GAPDH and 65% for LDH. The presence of antioxidants in the irradiated solution largely protected the enzymes from oxidation by thiol groups, with RSV found to be more effective than melatonin (Figure 4).

Changes in the secondary structure conformation of dehydrogenases

Circular dichroism spectroscopy was used to monitor changes in the secondary structure of the studied proteins following irradiation in the presence or absence of RSV or melatonin.

We compared changes in the secondary structure of GAPDH and LDH irradiated with X-ray doses of 210 and 420 Gy, respectively. Both enzymes were completely inactivated by the applied radiation doses. Figure 5 shows the far UV CD spectra of the two dehydrogenases following exposure to ionising radiation under the aforementioned conditions. Noteworthy changes in the CD spectra were only observed for LDH (bold values in Table 2). The secondary

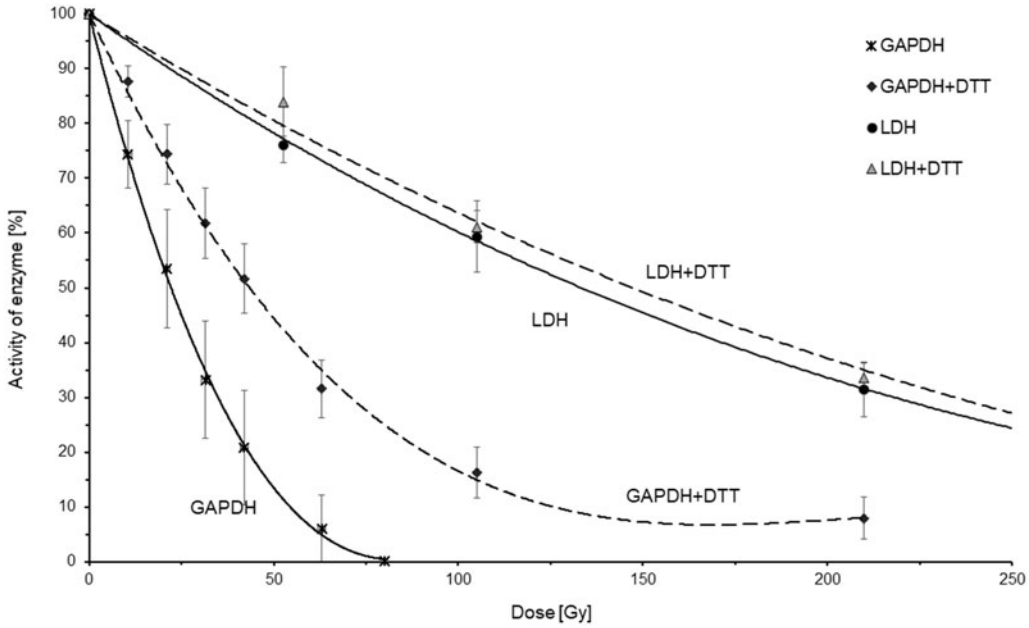


Figure 3. Inactivation of GAPDH and LDH upon exposure to radiation-induced reactive oxygen species. The activity of enzymes was determined after irradiation of the enzyme solution (solid line) or after incubation of the irradiated enzymes with the thiol reagent dithiothreitol (DTT) (dashed line).

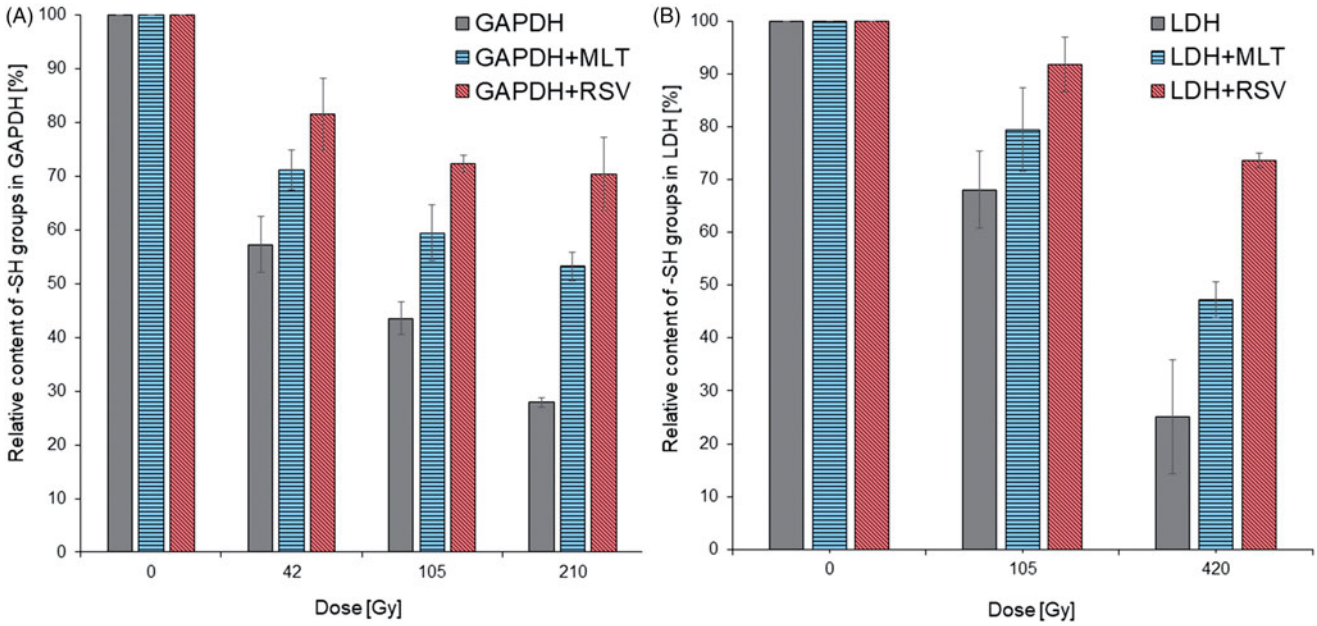


Figure 4. The relative content of sulfhydryl groups in GAPDH (A) or LDH (B) irradiated in the absence or presence of resveratrol or melatonin. The results are expressed as the means and standard deviations of 3–5 independent experiments. In all cases, statistically significant changes in the content of sulfhydryl groups were observed ($p < .05$).

structure of LDH is mostly made up of α-helices (approximately 40%). For the dose of 420 Gy, the content of α-helices decreased to 29.7%, while that of β-sheets and random coils increased (bold values in Table 2). The presence of RSV during the irradiation of LDH was found to completely protect the enzyme from secondary structure alterations. In this regard, MLT was less effective.

While GAPDH was completely inactivated at 210 Gy, no significant changes in its secondary structure were observed. This means that inactivation proceeded more rapidly than secondary structure modification at this dose (Table 2).

High-performance liquid chromatography (HPLC) separation of irradiated enzyme preparations

The structure of the studied proteins was determined by HPLC using a ZORBAX Bio Series GF-250 column. The main protein peak was eluted at approximately 10.75 min (maximum) for GAPDH and 10.5 min for LDH, which corresponds to the protein tetramers. The peaks decreased proportionally to the radiation dose (Figure 6). Additionally, GAPDH chromatograms revealed a peak that was eluted at a retention time of approximately 15 min,

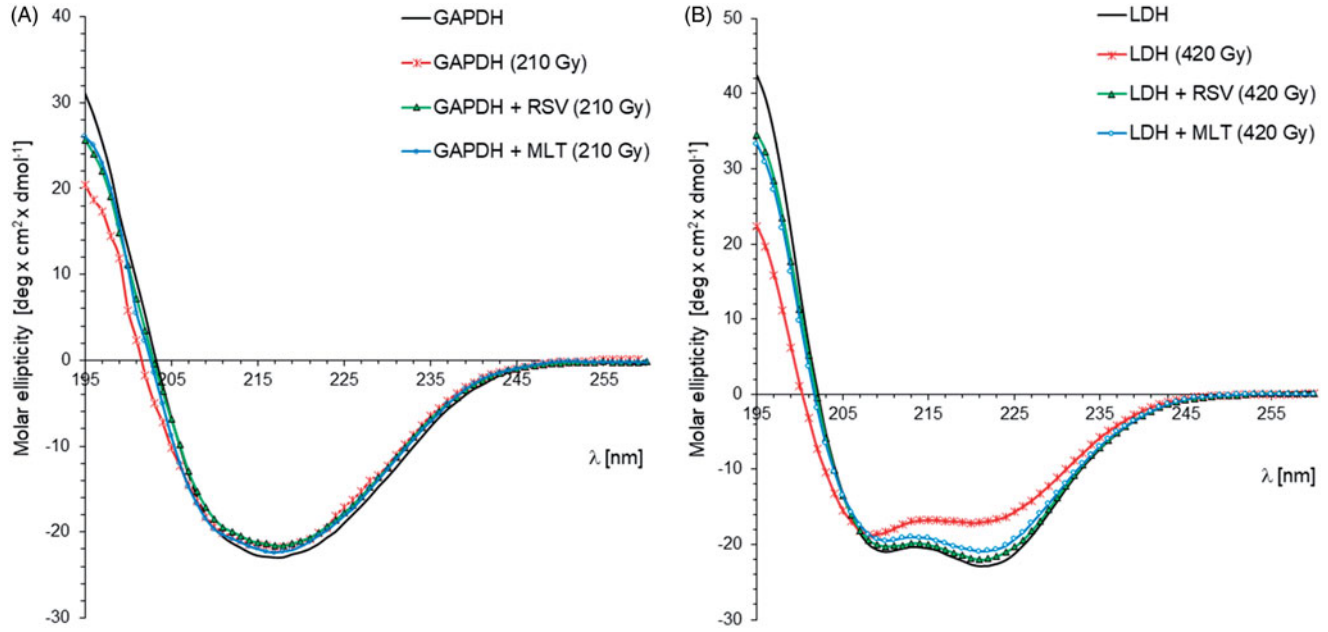


Figure 5. Far UV circular dichroism (CD) spectra of native and irradiated GAPDH (A) and LDH (B). The enzyme solutions were irradiated in the absence or presence of resveratrol or melatonin. The data are expressed as the molar residue ellipticity.

Table 2. The percentage of secondary structure content of GAPDH and LDH exposed to ionising radiation in the absence or presence of resveratrol or melatonin.

	GAPDH				LDH			
	0 Gy	210 Gy			0 Gy	420 Gy		
	–	–	+RSV	+MLT	–	–	+RSV	+MLT
Structure	Secondary structure content [%]							
α-Helices	30.9	29.3	31.5	31.5	**39.0**	**29.7**	38.4	33.3
β-structures	35.7	37.1	35.3	35.3	**30.2**	**37.0**	30.7	34.1
Random coil	36.1	35.7	35.0	35.0	**29.7**	**34.1**	29.7	33.0

The secondary structure content was calculated using CDNN software. The results summarised in the table are the means of 3–5 independent experiments. In all cases, the standard deviation was less than 0.5%.

corresponding to the enzyme monomers, which increased with the radiation dose (Figure 7).

The reduction in the tetramer peak was much lower for enzymes irradiated in the presence of RSV or MLT. Figure 8 shows the HPLC chromatograms of irradiated and non-irradiated GAPDH with and without RSV. In the presence of RSV, the tetramer peak decrease was much smaller compared to that of the enzyme irradiated in the absence of RSV. In the case of non-irradiated GAPDH in the presence of RSV, the maximum of the main tetramer peak was slightly shifted to the right, indicating that it was eluted at a longer retention time. This can probably be attributed to the fact that the complex formed from the binding of RSV to the protein is more hydrophobic. As previously mentioned, RSV exhibits very strong hydrophobic interactions with the column packing to which it adsorbs.

In the case of enzymes irradiated in the presence of RSV or MLT, the chromatograms revealed radiation-induced oxidation products of these antioxidants at retention times of over 15 min. In irradiated GAPDH preparations with RSV, the elution peak of RSV oxidation products occurred at approximately 22–23.5 min, accompanied by a slightly lower peak at about 20 min (Figure 8). These peaks roughly correspond to the peaks obtained during HPLC separation of an RSV solution irradiated in the absence of the enzyme.

Decreases in the tetramer fraction were quantified for the studied enzymes based on the area under the peak. The presence of RSV or MLT during irradiation was found to significantly reduce this decrease. The radiation yield determined for tetramer fraction degradation was 7.36×10^{-3} μmol/J for GAPDH irradiated in the absence of antioxidants, and 2.71×10^{-3} and 1.81×10^{-3} μmol/J for solutions irradiated in the presence of resveratrol and melatonin, respectively (Table 3). Comparatively, RSV protected LDH from degradation to a much greater extent. The radiation yield of tetramer fraction degradation for LDH preparations irradiated in the absence of ROS scavengers was 4.69×10^{-3} μmol/J, and this value declined to approximately 0.35×10^{-3} and 1.33×10^{-3} μmol/J for preparations containing RSV and MLT, respectively (Table 3).

Discussion

This work investigated the influence of resveratrol and melatonin on the structural and functional changes of two enzymes, LDH and GAPDH, under exposure to radiation-induced reactive oxygen species. These enzymes are widespread in living cells, and are both involved in the glycolytic pathway. GAPDH is a multifunctional enzyme, and modifications in this protein have been linked to a variety of pathologies (Seidler 2013; Sirover 2017). Moreover, GAPDH is among the proteins that are most vulnerable to oxidative and nitrosyl alterations in cells (Brandes et al. 2009; Dahl et al. 2015). It is present at very high concentrations in both eukaryotic and prokaryotic cells, accounting for 10% to 20% of total cell proteins (Seidler, 2013). While the studied

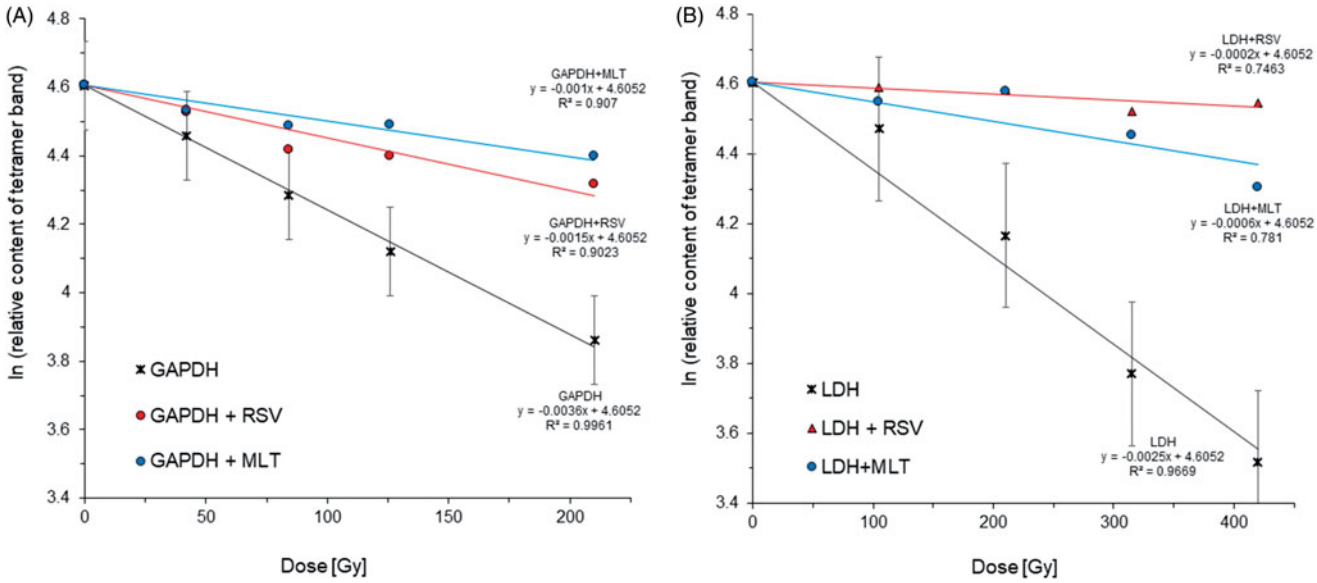


Figure 6. Degradation of a tetrameric fraction of GAPDH (A) and LDH (B) irradiated in the absence or presence of resveratrol and melatonin.

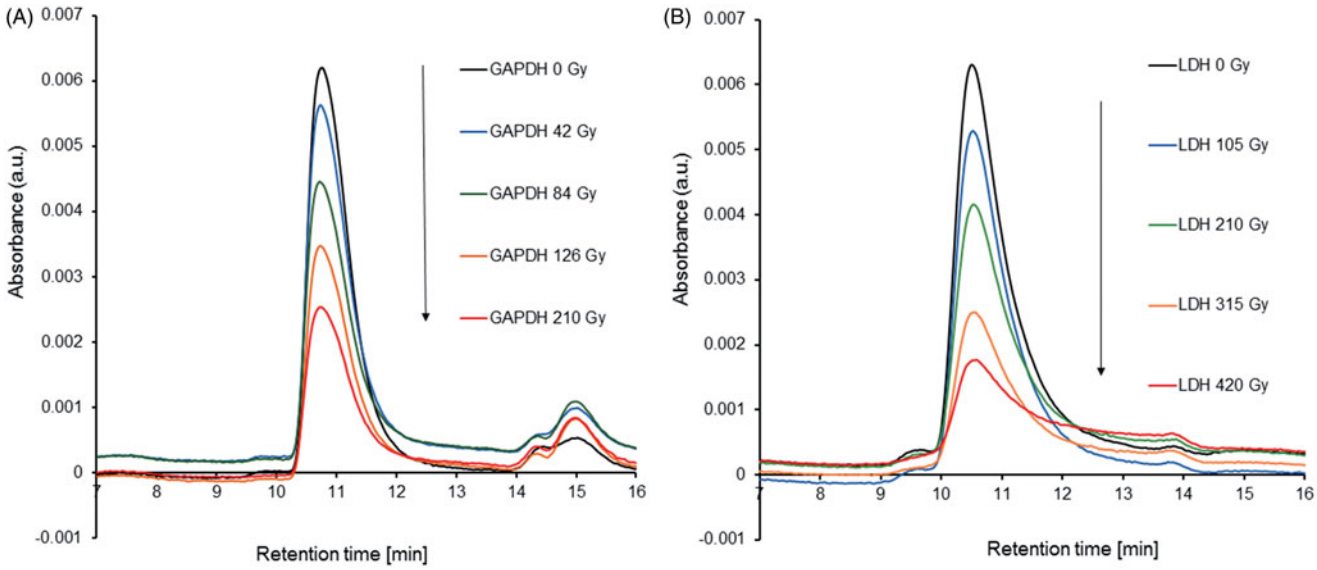


Figure 7. The high-performance liquid chromatography (HPLC) chromatograms of irradiated and non-irradiated aqueous solutions of GAPDH (A) and LDH (B), with UV detection at 278 nm.

proteins are structurally similar, except for their active sites, a previous study by our group found substantial differences in terms of radiosensitivity (Rodacka et al. 2010, 2012). Thus, the next logical step was to investigate the effects of low-molecular-weight antioxidants on radiation-induced structural and functional changes in these enzymes. Among the different water radiolysis products, hydroxyl radicals are the most efficient in terms of oxidative protein damage.

Rate constants for the reactions of $^{\bullet}OH$ and $O_2^{\bullet-}$ with the proteins, resveratrol and melatonin are presented in Table 4. Taking into consideration the concentration of the reagents during irradiation, we can calculate the percentage of hydroxyl radicals and $O_2^{\bullet-}$ reacting with the protein in competition with RSV or MLT (Table 5). When GAPDH or LDH were irradiated in the presence of RSV, 44% and 46% of the generated $^{\bullet}OH$ radicals reacted with the GAPDH and LDH, respectively and the remaining $^{\bullet}OH$ reacted with RSV. In contrast, $O_2^{\bullet-}$ radicals react with GAPDH only in 2% and in as much 98% with RSV. Nascent secondary RSV radicals cause enzyme inactivation upon reaction. In solutions irradiated in the presence of melatonin, 25% of the formed $^{\bullet}OH$ radicals and 98% $O_2^{\bullet-}$ reacted with the GAPDH, remaining radicals reacted with melatonin, producing secondary $^{\bullet}MLT$ radicals. To simplify the radiation-induced structural and functional changes of the studied proteins, we characterised them with the following reactions:

Irradiated with RSV

$$\text{GAPDH} + {}^{\bullet}\text{OH}(45\%) \rightarrow \text{GAPDH damage}$$

$$\text{GAPDH} + O_2^{\bullet-}(2\%) \rightarrow \text{GAPDH damage}$$

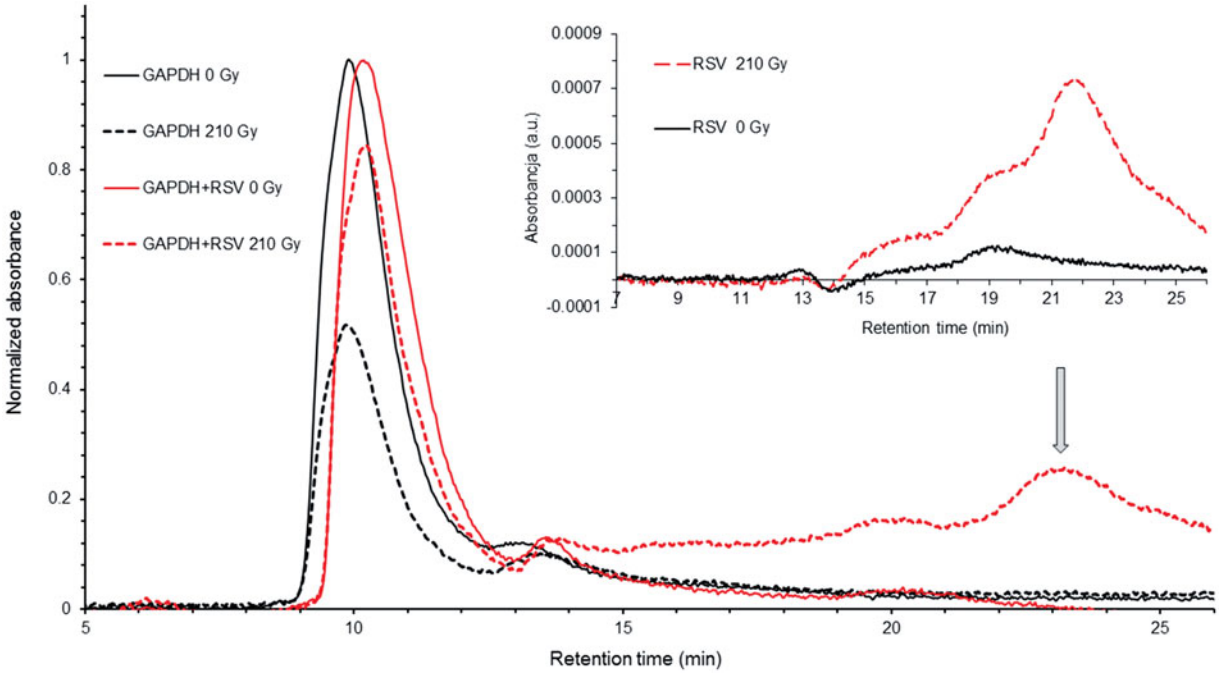


Figure 8. High-performance liquid chromatography (HPLC) chromatograms of irradiated and non-irradiated aqueous solutions of GAPDH in the absence or presence of resveratrol, with detection at 278 nm. Insert shows the HPLC chromatograms of aqueous solutions of non-irradiated and irradiated resveratrol, with detection at 278 nm.

Table 3. Radiolytic yields of reduction (G) of a tetrameric fraction of GAPDH and LDH irradiated in the absence or presence of resveratrol and melatonin.

Antioxidant	GAPDH $G \times 10^3$ (μmol/J)	LDH $G \times 10^3$ (μmol/J)
–	7.36 ± 0.45	4.69 ± 0.41
RSV	2.71 ± 0.24	0.35 ± 0.04
MLT	1.81 ± 0.16	1.33 ± 0.12

$$\mathrm{RSV} + {}^{\bullet}\mathrm{OH}(55\%) \rightarrow \mathrm{RSV}^{\bullet}$$

$$\mathrm{RSV} + \mathrm{O_2}^{\bullet-}(98\%) \rightarrow \mathrm{RSV}^{\bullet}$$

$$\mathrm{GAPDH} + \mathrm{RSV}^{\bullet} \rightarrow \mathrm{GAPDH\ damage}$$

Irradiated with MLT

$$\mathrm{GAPDH} + {}^{\bullet}\mathrm{OH}(25\%) \rightarrow \mathrm{GAPDH\ damage}$$

$$\mathrm{GAPDH} + \mathrm{O_2}^{\bullet-}(\sim 100\%) \rightarrow \mathrm{GAPDH\ damage}$$

$$\mathrm{MLT} + {}^{\bullet}\mathrm{OH}(75\%) \rightarrow \mathrm{MLT}^{\bullet}$$

$$\mathrm{GAPDH} + \mathrm{MLT}^{\bullet} \rightarrow \mathrm{GAPDH\ damage}$$

In the case of irradiation in the presence of melatonin, the reaction $\mathrm{O_2}^{\bullet-}$+MLT was neglected, due to the low reaction rate constant.

Corresponding reactions can also be applied to LDH.

The present study confirmed that GAPDH is about 6.7-times more radiosensitive to inactivation by water radiolysis products than LDH (Table 1). Melatonin and resveratrol protected both dehydrogenases from inactivation to different degrees. In the presence of RSV or MLT, radiation-induced GAPDH inactivation was 2.3- and 1.3-times lower, and LDH inactivation was more than 5.5- and 3.8-times lower, respectively (Table 1). Therefore, melatonin protected enzymes to a considerably lesser extent. This finding suggests that the formed melatonin radicals ($^{\bullet}$MLT) are much more efficient in protein inactivation compared to resveratrol radicals ($^{\bullet}$RSV). This is particularly evident for GAPDH, as irradiation in the presence of melatonin caused almost the same level of inactivation as that seen for samples without the antioxidant. As up to 75% of $^{\bullet}$OH radicals were replaced by secondary $^{\bullet}$MLT radicals, it can be concluded that $^{\bullet}$MLT are almost as equally efficient as $^{\bullet}$OH radicals in the inactivation of GAPDH. The higher protection against GAPGH inactivation by resveratrol is also due the fact that RSV is a much better scavenger of $\mathrm{O_2}^{\bullet-}$, which also contribute to GAPDH inactivation. It is important to bear in mind, however, that both antioxidants are much more efficient in the protection of LDH, and the effectiveness of $^{\bullet}$RSV and $^{\bullet}$MLT do not differ significantly in this regard.

Table 4. Rate constants of reactions of $^{\bullet}$OH and $\mathrm{O_2}^{\bullet-}$ with the enzymes, resveratrol and melatonin, and concentrations of the compounds employed in the experiments.

Reaction	Rate constant of reaction k [$\mathrm{mol^{-1}\ dm^3\ s^{-1}}$]	Concentration of the compound c [$\mathrm{mol\ dm^{-3}}$]
GAPDH+$^{\bullet}$OH	2.0×10^{11} [a,c]	2×10^{-6}
GAPDH + $\mathrm{O_2}^{\bullet-}$	2.0×10^{7} [b]	2×10^{-6}
LDH+$^{\bullet}$OH	2.1×10^{11} [c]	2×10^{-6}
LDH + $\mathrm{O_2}^{\bullet-}$	1.0×10^{5} [b]	2×10^{-6}
RSV+$^{\bullet}$OH	5.0×10^{9} [d]	1×10^{-4}
RSV + $\mathrm{O_2}^{\bullet-}$	2.0×10^{7} [d]	1×10^{-4}
MLT+$^{\bullet}$OH	1.2×10^{10} [e]	1×10^{-4}
MLT + $\mathrm{O_2}^{\bullet-}$	1.0×10^{4} [f]	1×10^{-4}

a – Kowalczyk et al. (2008); *b* – Bielski et al. (1985); *c* – Buxton et al. (1988); *d* – Mahal and Mukherjee (2006); *e* – Mahal et al. (1999); *f* – Roberts et al. (1998).

Table 5. The percentage of •OH radicals and $O_2^{\bullet-}$ radical that reacted with the protein in competition with resveratrol or melatonin (ω).

Irradiated solutions	The percentage of •OH radicals reacting with the protein in competition with the scavenger (ω)	The percentage of $O_2^{\bullet-}$ radicals reacting with the protein in competition with the scavenger (ω)
GAPDH	100	100
GAPDH + RSV	44	2
GAPDH + MLT	25	98
LDH	100	100
LDH + RSV	46	≈0
LDH + MLT	26	17

The calculation was made based on the concentration of the compounds used and their rate constants for reactions with •OH or $O_2^{\bullet-}$ radicals (*R*).

$$\omega(\text{enzyme} + {}^{\bullet}\text{R})(\%) = \frac{k({}^{\bullet}\text{R} + \text{enzyme}) \times [\text{enzyme}]}{k({}^{\bullet}\text{R} + \text{enzyme}) \times [\text{enzyme}] + k({}^{\bullet}\text{R} + \text{antioxidant}) \times [\text{antioxidant}]} \times 100$$

The differences in susceptibility to inactivation between LDH and GAPDH are primarily attributable to the structure of their active sites. GAPDH is a thiol-dependent enzyme, and the cysteine residue at position 149 is directly involved in catalytic reactions. This particular residue is characterised by a low pKa, meaning that the sulfhydryl group exists in a highly reactive nucleophilic thiolate anion ($R\text{-}S^-$) at physiological pH, and therefore reacts with oxidants faster than the protonated form (R-SH) (Rodacka et al. 2016).

When GAPDH was irradiated in the presence or absence of RSV or MLT incubated with DTT, its catalytic activity was found to be largely restored (Figure 3 and Table 1). This is due to reduction of the oxidised cysteine residue at the active site. In contrast, even though LDH contains one more cysteine per monomer than GAPDH, none of those residues are directly involved in enzymatic reactions. Therefore, the incubation of irradiated LDH with DTT did not significantly restore its catalytic activity, which indicates that oxidative modification of cysteine residues is not critical for its catalytic activity.

The HPLC experiments described herein show that while the radiation-induced destruction of GAPDH was only 1.6-fold higher than that of LDH, inactivation of the GAPDH was as much as 6.7-times greater than that of LDH. This can be attributed to the aforementioned presence of reactive –SH groups at the GAPDH active site, and their participation in catalytic reactions. RSV protected LDH from radiation-induced destruction to a much greater extent than GAPDH (13-fold vs. 2.7-fold), while MLT offered a similar level of protection in both cases (Table 3). Similar to protein inactivation, •MLT radicals produced more structural changes in the studied proteins than •RSV radicals.

In our previous work, we found that as far as water radiolysis products are concerned, LDH is inactivated to the greatest extent by hydroxyl radicals. At the concentrations found in our system, neither superoxide radicals nor hydrogen peroxide significantly affected LDH activity (Morgan et al. 2002; Rodacka et al. 2010). Thus, by scavenging the hydroxyl radicals generated as a result of LDH irradiation, RSV and MLT protect the enzyme from destruction and inactivation. Analysis conducted using bioinformatics tools revealed that RSV and MLT bind to LDH in greater amounts than GAPDH. While GAPDH can potentially bind 10 RSV and 18 MLT molecules on its surface, the corresponding numbers for LDH are 27 and 40. The affinity of ligands for the protein is characterized by the Dock-Score. The values of this parameter were in the range of 25–50 kcal/mol, with a mean value of about 34 kcal/mol, independently of protein and ligand. It can be concluded, therefore, that the affinity of ligands for the individual binding sites at the protein surface is similar and the actual number of bound antioxidant molecules is proportional to the number of potential binding sites (Figure 9).

Resveratrol and melatonin molecules cover parts of the surface of the studied proteins, thereby shielding them from direct attack by hydroxyl radicals. On the other hand, the •MLT and •RSV radicals arising from attacks by hydroxyl radicals, which are bound in the enzyme surface pockets, may oxidise the enzymes leading to inactivation and/or destruction. For the studied dehydrogenases, bioinformatics analyses indicate that there are a greater number of potential binding sites for MLT than RSV due to structural differences between the two antioxidants. The melatonin molecule has a very flexible side chain which can adopt various conformations, and readily interacts with proteins to form hydrogen and hydrophobic bonds. Similar to resveratrol, melatonin conferred better protection to LDH than GAPDH as this dehydrogenase can bind more melatonin molecules. At the same time, the degree of protection offered by melatonin to GAPDH is lower than that afforded by resveratrol, which suggests that •MLT radicals bound to GAPDH inactivate the enzyme more strongly than •RSV radicals.

Under the influence of ionising radiation, resveratrol and melatonin undergo radical oxidation processes. Camont et al. (2012) and Bonnefont-Rousselot et al. (2011) reported that the main (stable) products of radiation-induced oxidation of RSV in the presence of oxygen (•OH/ $O_2^{\bullet-}$) are 3,5-dihydroxybenzoic acid (3,5-DHBA) and piceatannol, while the major MLT oxidation product is N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK). Based on these publications, it is possible to quantify the major oxidation products of melatonin and resveratrol. Irradiation of a 100 μmol dm^{-3} RSV solution with a dose of 210 Gy yields 20.2 μmol dm^{-3} 3,5-DHBA and 6.7 μmol dm^{-3} piceatannol. Under the same experimental conditions, radiation-induced melatonin oxidation gives 51.2 μmol dm^{-3} AFMK. Thus, the question arises as to whether the oxidation products of RSV and melatonin affect the activity of the studied dehydrogenases. To

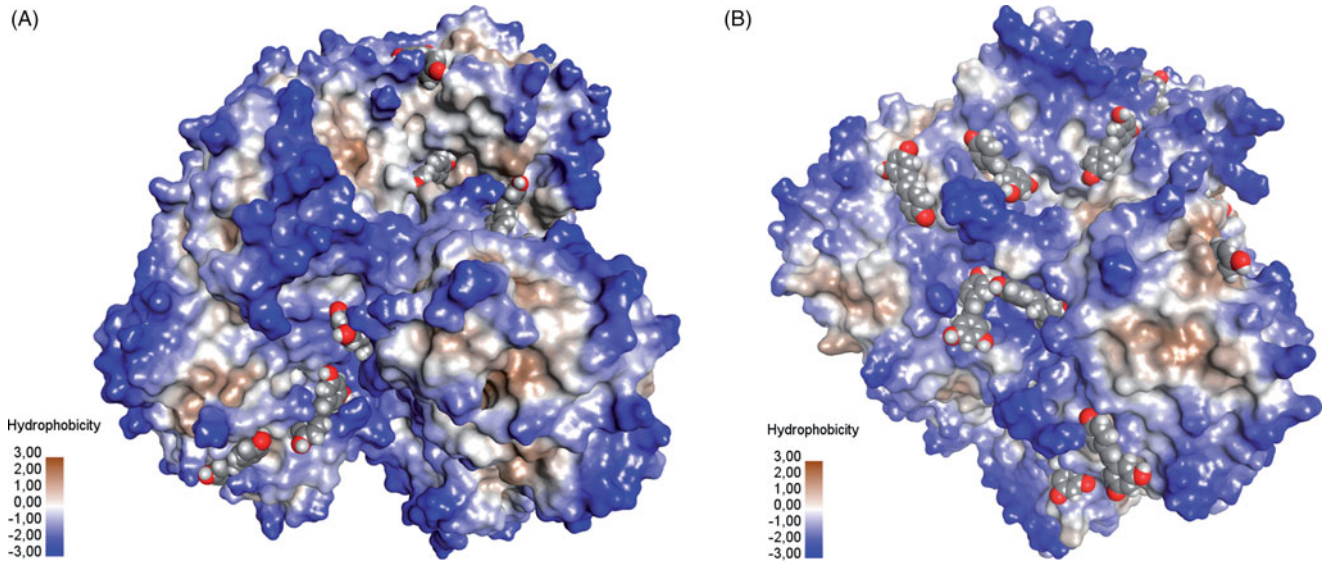


Figure 9. Molecular docking of GAPDH (A) and LDH (B) with resveratrol. The protein surface is coloured according to its hydrophobicity, with a hydrophilic (blue) to hydrophobic (light brown) gradient.

investigate this, GAPDH and LDH were exposed to the main oxidation products of resveratrol (3,5-DHBA and piceatannol) and melatonin (AFMK) for 60 min, after which the enzymatic activities were measured. At concentrations corresponding to those generated by 210 Gy, piceatannol, 3,5-DHBA and AFMK did not inactivate GAPDH. The activity of LDH was not affected either, even at double the concentrations (corresponding to irradiation with a dose of 420 Gy).

During the irradiation of resveratrol and melatonin solutions in the presence of proteins, the resulting radical products of the low-molecular-weight compounds were found to directly react with the protein molecules. Therefore, the oxidation products of RSV and MLT may differ, and if they do arise, they are present in much lower amounts. HPLC separation of proteins irradiated in the presence of resveratrol or melatonin revealed the presence of oxidation products of both low-molecular-weight compounds (a sample chromatogram is presented in Figure 8). These products are eluted at almost the same retention times (over 15 min) as those obtained following melatonin and RSV irradiation in the absence of proteins.

Summary and conclusions

The findings clearly indicate that resveratrol and melatonin differ in the degree of protection they provide GAPDH and LDH enzymes from radiation-induced destruction and inactivation.

1. Compared to melatonin, resveratrol is more effective at protecting the studied enzymes from inactivation, with a higher degree of protection for LDH (5-fold) than GAPDH (2.3-fold). This difference can be explained by the presence of reactive –SH groups in the GAPDH active site, and the fact that it is also inactivated by secondary melatonin radicals and, to a lesser extent, resveratrol radicals. On the other hand, LDH does not contain –SH groups in its active site, and it is not inactivated by water radiolysis products other than hydroxyl radicals and it is not inactivated by secondary radicals of $^{\bullet}$RSV or $^{\bullet}$MLT. Moreover, the higher protection of GAPDH by RSV in comparison with MLT is partly due to the higher effectiveness of scavenging of $O_2^{\bullet -}$ radicals by resveratrol (Table 4). In addition, it should be kept in mind that melatonin radicals inactivate GAPDH more strongly than resveratrol radicals.
2. Resveratrol protected the structure of LDH to a greater extent (13-fold, as determined by HPLC) than GAPDH (2.7-fold). This difference can be attributed to the fact that LDH binds many more RSV molecules (27 binding sites) than GAPDH (10 binding sites). Binding of RSV to LDH shields the protein surface from attack by hydroxyl radicals. This is also corroborated by the complete protection of the LDH secondary structure by RSV

In summary, the results of this study demonstrate that resveratrol has better antioxidant properties toward GAPDH and LDH when compared to melatonin.

Disclosure statement

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The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH

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ABSTRACT

Background: Nitric oxide is a well-known gaseous signaling molecule and protein modifying agent. However, at higher concentrations or during oxidative stress nitric oxide may exert some deleterious effects on protein structure and function. Here we investigated the influence of nitric oxide and products of its oxidation on two glycolytic enzymes: GAPDH and LDH under in vitro nitrosative stress conditions. Secondly, we applied natural antioxidants: melatonin and resveratrol to examine their effects on the enzymes under studied conditions.

Methods: By means of UV–VIS and fluorescence spectroscopy methods we compared nitric oxide mediated changes of enzyme activities, amount of free sulfhydryl groups (-SH) and bis-ANS probe binding. Finally, we predicted potential cysteine residues modtified by nitric oxide in studied proteins using GPS-SNO software.

Results: Our results indicated that nitric oxide reversibly inactivates GAPDH but does not affect the activity of LDH. Nitric oxide dependent GAPDH activity decline was accompanied by the reduction of the amount of free –SH groups and GAPDH-bound bis-ANS fluorescence. Reduction of the number of free –SH groups and protein-bound bis-ANS fluorescence was also observed in LDH treated with NO. Applied antioxidants increased inactivation of GAPDH and structural changes of GAPDH and LDH.

Conclusions: Nitric oxide modifies function and structure of thiol-dependent enzyme such as GAPDH and structure of LDH which function do not rely on cysteine thiols. Both resveratrol and melatonin exerted prooxidative properties in studied conditions.

General significance: Extensively studied antioxidants: resveratrol and melatonin may function as a prooxidative species under in vitro nitrosative stress conditions.

1. Introduction

Oxidative protein modifications may alter protein functions and are often a hallmark of a specific disease state [1–4]. Nitric oxide (NO) plays a crucial role in the reactive nitrogen species (RNS) mediated oxidative protein modifications. This free radical exerts its primary signaling function such as activation of guanylate cyclase and nascent vasodilatation in the reaction called nitrosylation in which it produces coordinative bounds with hem center of the enzyme [5–6]. Secondly, nitric oxide serves as a precursor molecule to other RNS. In the reaction with oxygen or reactive oxygen species (ROS) such as superoxide anion radical ($O_2\cdot^-$) it produces higher nitric oxides (NO_2, N_2O_3) or peroxynitrite ($ONOO^-$), respectively. These species together with nitric oxide itself are able to modify specific amino acid residues (Cys or Tyr) in proteins [7]. Nitric oxide mediated oxidative, posttranslational protein modifications lead to changes in protein function (gain or loss of function), localization or protein-protein interactions. Moreover, modified proteins may serve as a storage and/or delivery vehicles of NO to other protein targets [8].

The mechanism by which nitric oxide and its oxides modify cysteine residues is S-nitrosylation (or S-nitrosation, this term is less frequently used but regarded as a proper chemical nomenclature). S-nitrosylation of protein residues is considered as an alternative or non-classical nitric oxide signaling pathway [9]. It is a reversible, chemical modification

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; bis-ANS, 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; DTT, dithiothreitol; GSH, glutathione reduced; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DMSO, dimethyl sulfoxide; RSV, trans-3,5,4′-trihydroxystilbene (resveratrol); MEL, *N*-acetyl-5-methoxytryptamine (melatonin)

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where the structure of a target protein is of paramount importance due to its specificity [10–14]. However, during nitrosative stress, specificity of S-nitrosylation is lost and cysteines which normally are not modified also become S-nitrosylated. It results in changes of protein function or even leads to protein aggregation [9]. Aberrant protein S-nitrosylation connected with nitrosative stress is a hallmark of two most common neurodegenerative diseases, namely Parkinson's and Alzheimer's diseases [15–17].

One of the proteins that play a particular role in above mentioned disorders, during oxidative/nitrosative stress is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [18]. GAPDH is an enzyme involved in glucose metabolism during glycolysis but has a broad range of functions unrelated to glycolysis. For this reason it is regarded as a multifunctional or moonlighting protein [19,20]. GAPDH consists of four equal approximately 37 kDa monomers forming homotetrameric quaternary structure. It is also a redox sensitive protein, prone to oxidative modifications. Owing to nitrosative stress GAPDH becomes S-nitrosylated at Cys-149 (rabbit GAPDH) or Cys-151 (human GAPDH) residue critical to its glycolytic function [21–23]. Modified GAPDH interacts with Siah 1 ubiquitin ligase and translocates to nucleus, especially in neuronal cells [24]. As a result of nuclear translocation such protein complex initiates Siah 1 dependent nuclear proteins degradation and apoptosis [24].

In addition, there are several studies indicating that nitric oxide mediated GAPDH cysteine modifications result in conformational changes of a protein through disulfide bond formation that leads to its amyloid-like fibril formation [21,22]. Proapoptotic and proaggregation properties of oxidatively modified GAPDH during nitrosative stress are two hallmarks of GAPDH mediated neurodegeneration.

As stated above, the main targets of NO in proteins are thiol groups. It can be expected therefore that proteins having functional thiol groups should be more susceptible to the action of NO. The aim of this paper was verification of this hypothesis by comparison of NO sensitivity of two structurally similar dehydrogenases, one having reactive cysteine residue group in the active site (GAPDH) and another not relying on thiol group in its catalytic activity (LDH) [25–28]. Another problem investigated concerned the possibility of protection against NO-induced protein modification by antioxidants. As thiol antioxidants react with NO, we studied the effect of two potent non-thiol antioxidants: resveratrol and melatonin. Both of selected antioxidants easily cross blood-brain barrier and may potentially protect neurons from GAPDH mediated neurodegeneration [29,38].

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenol found in grapes, red wine, basil, artichoke and mulberries [29,30]. This stilbene has pleiotropic effects that include cardioprotective, anti-inflammatory, chemopreventive and neuroprotective properties [31,29]. The best known cardioprotective effect of resveratrol is in part due to polyphenol mediated upregulation of endothelial nitric oxide synthase (eNOS) expression and activity. Moreover, resveratrol indirectly diminishes the level of ROS and prevents superoxide anion radical mediated decomposition of NO produced by eNOS [32]. In the central nervous system resveratrol acts as a neuroprotective agent directly scavenging free radicals, stimulating activity of several antioxidant enzymes (catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR)) or downregulating the expression of inducible nitric oxide synthase (iNOS) which prevent nitrosative stress [33].

Melatonin (*N*-acetyl-5-methoxytryptamine) is another naturally occurring antioxidant. This indoleamine is a hormone product of pineal gland in mammals, but it is produced by other animals and plants as well [34]. Thanks to its circadian rhythm regulatory properties it is often used as a supplement by humans. Melatonin is also present in several plant dietary sources such as seeds (sunflower, anise, mustard, wolfberry and oat), fruits (banana) and olive oil [35]. Other equally important features of this compound are direct and indirect antioxidant properties. Melatonin is a good electron donor that easily scavenges hydroxyl radical, nitric oxide and peroxynitrite [36]. Similar to resveratrol, melatonin increases activity of several antioxidant enzymes such as SOD, GR and glutathione peroxidase (GPx) and decreases prooxidative activity of iNOS. For these reasons it is regarded as a therapeutic agent in many conditions connected with oxidative stress including neurodegenerative disorders [34,37,38].

2. Materials and methods

2.1. Materials

Studied proteins: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH) from rabbit muscle were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Hemoglobin (Hb) was isolated from human red blood cells and further purified by ion-exchange chromatography on carboxycellulose resin in the 0.02 M sodium phosphate buffer pH (6.7–8.0) gradient [39]. All other chemicals were purchased from Sigma-Aldrich, Avantor Performance Materials Poland S.A or as specified.

2.2. Methods

2.2.1. Nitric oxide generating apparatus and preparation of nitric oxide working solution

Gaseous nitric oxide was produced using a modified method of Opländer et al. [40]. In this method nitric oxide is generated by the photolytic decomposition of nitrite solution in the presence of ascorbate. The initial nitric oxide generating solution contained: 0.5 M sodium nitrite and 0.04 M ascorbate in 0.01 M sodium phosphate buffer pH 7.4 and was purged at 25 °C with inert gas (argon) for 1 h. In the separate glass column solution of 0.01 M sodium phosphate buffer pH 7.4 was degassed with argon. To initiate nitric oxide production, reaction chamber was irradiated with UVA light for 10 min at 25 °C. After that time nascent nitric oxide was transported using moderate flux of argon to the column containing degassed phosphate buffer for 5 min at room temperature. During this step temperature in reaction chamber was elevated to 40 °C.

2.2.2. Determination of nitric oxide concentration

To determine the exact concentration of generated nitric oxide we applied spectrophotometric assay. The assay is based on the nitric oxide induced conversion of hemoglobin ($Hb(Fe^{2+})O_2$) to methemoglobin Hb (Fe^{3+}) according to the reaction (1) [41]:

$$NO + Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (1)$$

To estimate the concentration of formed nitric oxide after each generation reaction we prepared a standard curve. Different volumes of buffer containing NO were added to the solution of approximately 0.8 mg ml^{-1} Hb followed by absorbance measurement at 576 nm using 10 mm path length cuvette in a Cary1 UV–visible spectrometer (Varian, Australia). We estimated percent of formed methemoglobin (metHb) according to the Eq. (2):

$$\%\text{metHb} = \left(\frac{A_1 - A_2}{A_1 - A_3}\right)\cdot 100\% \quad (2)$$

where: A_1 is a absorbance at 576 nm of the control sample of Hb, A_2 is a absorbance at 576 nm of the Hb with a known volume of NO containing buffer, A_3 is a absorbance at 576 nm of metHb induced by the addition of potassium hexacyanoferrate (III) to the control sample.

Using molar extinction coefficient of Hb of 14,600 M^{-1} cm^{-1} and calculating percent of metHb formed from standard curve we were able to determine the concentration of produced metHb. Concentration of formed metHb was regarded as equal to the concentration of NO added to the measured samples and served to define the nitric oxide concentration in whole volume of working buffer for further study.

2.2.3. Preparation of enzymes and antioxidants solutions

GAPDH and LDH were prepared by dissolving lyophilized protein powder in 0.1 M phosphate buffer pH 7.4. Concentrations of the enzymes were estimated spectrophotometrically using extinction coefficients of $A_{280}^{1\%} = 10$ for GAPDH and $A_{280}^{1\%} = 14.9$ for LDH, respectively. Resveratrol was prepared at the concentration of 20 mg ml^{-1} in DMSO and diluted in 0.01 M phosphate buffer pH 7.4 to the required concentration prior to use. Resveratrol at the final concentration used in the experiments does not affect protein activity [42]. Melatonin ex substantia was dissolved in 0.01 M phosphate buffer pH 7.4 and the exact concentration of the working solution was estimated spectrophotometrically using molar extinction coefficient of 6.3 mM^{-1} cm^{-1} at 278 nm.

2.2.4. Activity assay of GAPDH exposed to nitric oxide in the presence or absence of antioxidants

2 μM solution of GAPDH exposed to different concentrations of NO in the presence or absence of 50 μM of melatonin or resveratrol, respectively, was incubated for 1 h at room temperature in the dark. Activity of the enzyme was then estimated by measuring kinetics of NADH formation during oxidation of glyceraldehyde 3-phosphate to 1,3-bis-phosphoglycerate in the presence of arsenate ions as described previously [42]. The results were expressed as a percent relative to the control values of GAPDH activity.

2.2.5. Activity assay of LDH exposed to nitric oxide in the presence or absence of antioxidants

Activity of LDH was studied after 1 h incubation of 2 μM enzyme with increased concentrations of nitric oxide. Kinetics of the formation of NAD^+ from NADH during conversion of pyruvate to lactate catalyzed by LDH was monitored by measuring absorbance at 340 nm using modified methodology of Wroblewski and LaDue [43]. The velocity of such reaction and the activity of the enzyme were expressed as a percent of control values.

2.2.6. Determination of nitric oxide concentration by the analysis of its end products in aqueous solution in the presence of protein

The concentrations of NO metabolites in solution, NO_2^-/NO_3^-, were estimated with Nitrate/Nitrite Colorimetric Assay Kit (Item No. 780001, Cayman Chemical Company), based on reaction with the Griess reagent. The reaction was carried out in 96-well microplates. In order to determine the total NO_2^- and NO_3^- levels, NO_3^- was reduced for 1 h at room temperature by addition of Nitrate Reductase Mixture in the presence of Enzyme Cofactor Mixture. After the incubation time Griess Reagent 1 (sulfanilamide) was added followed by addition of Griess Reagent 2 (*N*-(1-naphthyl)ethylenediamine). Samples were incubated for 10 min and the absorbance was measured at 540 nm using Cary-50 spectrophotometer with a microplate reader (Varian, Australia). Nitrate or nitrite standard curves were prepared in the range of 0–35 μM using 200 μM standard solutions. Concentration of NO metabolites in protein solution was measured after exposure of 2 μM solution of GAPDH to either 26 μM or 103 μM initial NO concentration and 30 or 60 min incubation.

2.2.7. Effect of thiol reducing reagents (DTT, GSH) on the activity of GAPDH exposed to nitric oxide

2 μM solution of GAPDH with a specific concentration of NO was incubated for 30 min in the dark. Protein samples exposed to nitric oxide were then treated with the final concentration of either 4 mM GSH or 19 mM DTT. Activity assay of nitrosylated GAPDH exposed to the excess of reducing reagents was performed as described above at specific time intervals: 2, 5 and 20 min after addition of reducing reagents and expressed as a percent of control values.

2.2.8. Analysis of free sulfhydryl groups in the studied enzymes exposed to nitric oxide in the presence or absence of antioxidants

To assess the relative content of –SH groups in proteins exposed to nitric oxide we used the modified Ellman's method [44]. The free –SH content was determined under denaturating conditions using final concentration of 4.75 M guanidine hydrochloride (Gdn-HCl) in 0.1 M phosphate buffer pH 8.0 and expressed as a percent of control values. 2 μM solutions of the enzymes exposed to nitric oxide in the presence or absence of melatonin or resveratrol, respectively, were incubated in the dark for 1 h. Samples were then diluted using 6 M Gdn-HCl solution and incubated in the dark with final 99 μM concentration of DTNB for 10 min followed by absorbance measurements.

2.2.9. Structural changes of the studied proteins exposed to nitric oxide examined with fluorescence probe bis-ANS

Kinetics of bis-ANS binding to the studied proteins treated with NO in the absence or in the presence of investigated antioxidants were recorded at $\lambda_{exc} = 395$ nm and $\lambda_{em} = 495$ nm. The measurements were performed using thermostatted spectrofluorometer Cary Eclipse (Varian, Australia) at the temperature of 22 °C. The protein concentration was 2 μM and the ligand concentration was 50 μM prepared in sodium phosphate buffer (pH 7.4) as mentioned above. After treatment with NO (~150 μM), samples were first incubated for 45 min in the dark and then bis-ANS (40 μM) was added. The kinetics of fluorescence changes was recorded for 40 min. The fluorescence intensity read after 30 min from the start of the measurement was taken for further analysis. Changes in fluorescence of bis-ANS after binding to the protein treated with NO in the absence or the presence of studied antioxidants were expressed as a percent of control values.

2.2.10. Computational prediction of potential S-nitrosylation sites of rabbit GAPDH and rabbit LDH

To predict cysteine residues that can be modified by nitric oxide in both studied enzymes we employed GPS-SNO 1 software according to Xue et al. [45]. The studied sequences of rabbit GAPDH (P46406) and rabbit LDH (P13491) were analyzed using medium threshold as a default.

3. Results and discussion

3.1. Analysis of nitric oxide end products in the presence of a protein in aerated solution

Nitric oxide as a free radical easily reacts with other molecules containing unpaired electron in their valence shell. However, it is still a matter of debate whether NO itself is able to modify protein thiols or rather nitric oxide oxidation products are the real protein thiol modifying agents [46,47]. In our reaction system degassed buffer solution was purged with generated gaseous nitric oxide. The concentration of NO was determined in the reaction with oxyhemoglobin and NO was immediately added to the protein solution. Firstly, we analyze the reaction products of nitric oxide with oxygen in the presence of protein (GAPDH) (Table 1).

Nitric oxide in aerated water solution reacts with oxygen in sequential reactions [48–50]:

$$NO^{\cdot} + O2 \leftrightarrows OONO^{\cdot} \quad (3)$$

$$ONOO^{\cdot} + NO^{\cdot} \rightarrow 2\,NO_2^{\cdot} (k = 2 \times 10^6\ M^{-2}\ s^{-1}) \quad (4)$$

$$2\,NO_2^{\cdot} + 2NO^{\cdot} \leftrightarrows 2\,N_2O_3\ (k = 1.1 \times 10^9\ M^{-1}\ s^{-1}) \quad (5)$$

$$2\,N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+\ (k = 4.75 \times 10^4\ s^{-1}) \quad (6)$$

Consistent with Eq. (6) the final decomposition product of NO in water solution is nitrite. However, an alternative route exists where NO oxidation ends with both nitrite and nitrate (Eq. (7)). It starts with the dimerization of nitrogen dioxide (Eq. (4′)) followed by its

Table 1
Concentration of nitric oxide metabolites in protein solution estimated using Nitrate/Nitrite Colorimetric Assay Kit (Cayman). After addition of 0.0258 or 0.1030 mM NO in 0.01 M sodium phosphate buffer pH 7.4 to 2 μM GAPDH, samples were incubated for 30 min or 1 h, respectively, followed by the spectrophotometric measurement of nitrite and nitrate. The results represent the average from four replicates of a single experiment. Standard deviations were omitted for the sake of table clarity but they were < 10%.

C_{NO} [mM]	30 min				1 h			
	NO_2^- [mM]	NO_3^- [mM]	NO_2^-/NO_3^- total [mM]	NO_2^-/NO_3^- ratio	NO_2^- [mM]	NO_3^- [mM]	NO_2^-/NO_3^- total [mM]	NO_2^-/NO_3^- ratio
0.0258	0.378	0.0825	0.460	4.582	0.378	0.131	0.509	2.885
0.1030	1.135	0.479	1.614	2.370	1.031	0.577	1.608	1.787

decomposition to both nitrite and nitrate.

$$ONOO^{\cdot} + NO^{\cdot} \rightarrow 2\,NO_2^{\cdot} \rightarrow O_2NNO_2\,(N_2O_4) \quad (4')$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (7)$$

Several studies indicate that this route is of marginal importance in water solution where reaction (5) is dominant and nitrogen dioxide dimer (N_2O_4) is not present or present only in insignificant quantities [49]. The high nitrite/nitrate ratio in our reaction system (Table 1) indicates that this is also true in the presence of protein thiols. The total concentration of both nitrite and nitrate is from 16 to 20 times higher than the initial concentration of nitric oxide estimated in the reaction with oxyhemoglobin (Table 1). Taking into account the above stoichiometry of nitric oxide oxidation it clearly demonstrates that, oxidation process has already been initiated before addition of NO solution to protein samples.

3.2. Functional changes of studied dehydrogenases upon exposure to nitric oxide and its metabolites

We examined the functional changes of the enzymes (GAPDH and LDH) due to their reaction with nitric oxide (Fig. 1), by estimation of changes in their activities.

Nitric oxide based cysteine modifications of thiol dependent enzymes such as alcohol dehydrogenase (ADH) or rhodanese lead to their inactivation [51,52]. The mechanism of inactivation is mainly due to S-nitrosylation of active site cysteines. Our results also indicate nitric oxide dependent inhibition of GAPDH activity which is consistent with published data [23,53] (Fig. 1). Rabbit GAPDH contains four cysteine residues per monomer: Cys-149, Cys-153, Cys-244, Cys-281 and the active site Cys-149 which occurs as a thiolate anion at neutral pH is the most sensitive to oxidative modifications. It is also regarded as a primary target to NO donors in vitro which result in either S-nitrosylation or disulfide bond formation. The consequences of mentioned Cys-149 modifications are loss of catalytic function or protein aggregation, respectively [21–23,53]. Furthermore, close proximity of cofactor

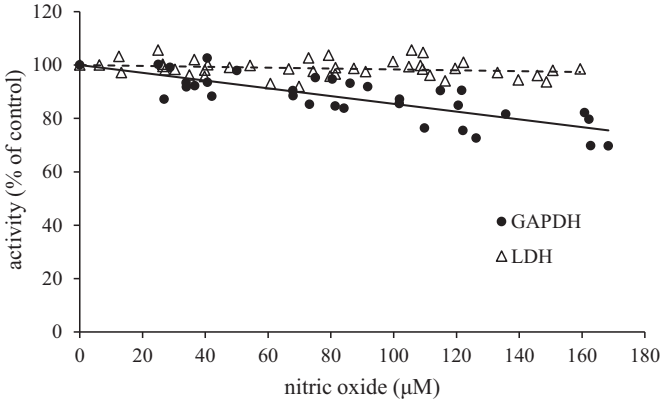

Fig. 1. Activity of GAPDH and LDH exposed to different concentrations of generated nitric oxide expressed as a percent of control. Prior to enzymes kinetics studies protein samples containing nitric oxide were incubated for 60 min in the dark.

binding pocket to S-nitrosylated Cys-149 enables transnitrosation of NAD(H) molecule and subsequent covalent attachment of GAPDH to nicotinamide ring of NAD(H) via Cys-149. Opposite to S-nitrosylation, this modification results in irreversible enzyme inhibition but may protect enzyme from nitrosative stress induced aggregation [23,54].

Several studies indicating that the meaning of GAPDH S-nitrosylation to the cell fate is more complex and encompasses different cellular compartments. Inactive S-nitrosylated GAPDH may change cellular localization and translocate to mitochondria or nucleus where it interacts with several proteins including: acetyl-Coenzyme A acetyltransferase 1 (ACAT1), 60 kDa heat shock protein (Hsp60), NAD-dependent deacetylase sirtuin-1 (SIRT1) and histone deacetylase-2 (HDAC2) [55,56]. In that manner S-nitrosylated GAPDH becomes a redox information hub that transduce NO signals to a specific protein targets through transnitrosylation process what results in changing of recipient protein function [57]. As it was studied in case of SIRT1 even a small amount of S-nitrosylated GAPDH in comparison to the total cell GAPDH content was able to efficiently inhibit enzymatic activity of nuclear SIRT1. Finally, apart from its function as a transnitrosylase, S-nitrosylated GAPDH forms many other protein complexes; including that with Siah1 ubiquitin ligase toxic to the cell and inducing cell death [24].

Our results indicate that contrary to GAPDH, LDH activity after exposure to nitric oxide remains unaffected (Fig. 1). Rabbit muscle LDH contains five Cys residues per monomer (Cys-34, Cys-130, Cys-162, Cys-184, Cys-292) all located inside the molecule [27]. As shown in Fig. 8 none of them is within active site of the enzyme and only one Cys-162 is potentially available for S-nitrosylation. These are probable reasons for high functional insensitivity of LDH to generated nitrosative stress.

There are two proposed mechanisms of protein cysteine thiol modifications with NO through S-nitrosylation. The first assumes S-nitrosylation of cysteine thiols with dinitrogen trioxide (Eq. (8)) and the second postulates radical-radical interaction where thiyl radicals formed according to Eq. (7) interact directly with nitric oxide radicals (Eq. (9′)) [50].

$$N_2O_3 + RSH \rightarrow RSNO + HNO_2\,(k = 6.6 \times 10^7\ M^{-1}\ s^{-1}) \quad (8)$$

$$RSH + NO_2^{\cdot} \rightarrow RS^{\cdot} + NO_2^- + H^+\,(k = 2.2 \times 10^7\ M^{-1}\ s^{-1}) \quad (9)$$

$$RS^{\cdot} + NO^{\cdot} \rightarrow RSNO\,(k = 3 \times 10^9\ M^{-1}\ s^{-1}) \quad (9')$$

Since in the presented study oxidation of nitric oxide is initiated when added to protein thus it seems that all of the protein nitrosylation scenarios are potentially possible.

3.3. The influence of nitric oxide on the amount of free thiols in the studied proteins. Restoration of enzyme activities by reducing agents

To further verify that loss of protein activity was mainly due to protein thiol inactivation we determined the amount of protein free thiols. We observed that GAPDH treated with the same concentration of NO retains less free –SH groups than LDH (Fig. 2). This conclusion is consistent with our GPS-SNO predictions where GAPDH contains more potential S-nitrosylation sites than LDH (Fig. 8).

Restoration of nitric oxide modified GAPDH activity with the use of two commonly known thiol reductants (DTT, GSH) confirms involvement of Cys-149 thiols in the process of nitric oxide mediated GAPDH

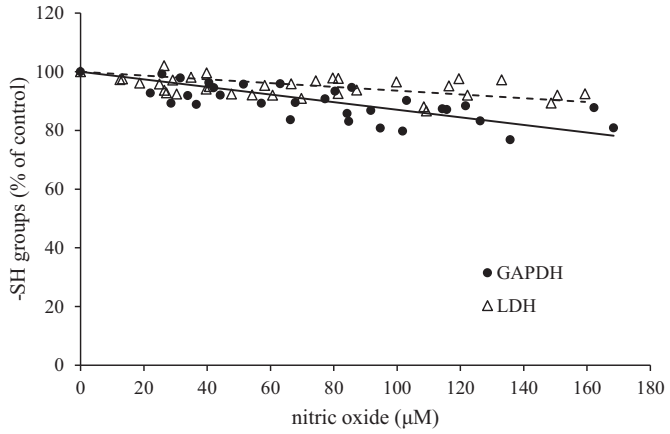

Fig. 2. Free –SH groups in GAPDH and LDH exposed to different concentrations of NO. Protein samples were incubated in the dark for 60 min followed by addition of DTNB to the final concentration of 99 μM and incubated for another 10 min. Thiol groups were determined by measuring the absorbance at 412 nm and expressed as a percent of control values.

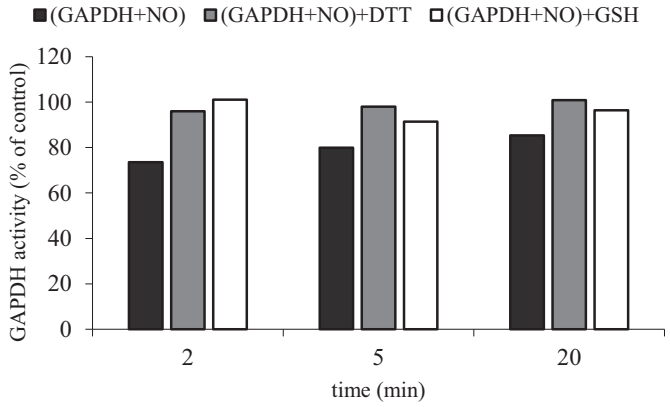

Fig. 3. Reducing agents restored activity of nitric oxide modified GAPDH. After exposure to nitric oxide (111.84 μM) GAPDH was incubated in the dark for 30 min followed by the addition of either 4 mM GSH or 19 mM DTT final concentration. Activity assay was performed 2, 5, 20 min after addition of reducing reagents. Data are expressed as a percent of control with appropriate reducing agent and represents the results of a single experiment.

functional changes. Both reductants are equally efficient and are able to restore GAPDH activity almost immediately (2 min) after addition to protein samples (Fig. 3). Comparable results were also obtained for higher GAPDH concentration and for ADH (data not shown). The reversibility of GAPDH S-nitrosylation with the use of reducing agents was also observed with similar experiments of Mohr et al. [23] and Zaffagnini et al. [53].

3.4. The influence of melatonin on the function of GAPDH and LDH in the presence of nitric oxide

Melatonin increased inactivation of GAPDH caused by nitric oxide from about 20% (Fig. 1) to about 50% (Fig. 4) at the NO concentration of 170 μM. However, this effect was not observed in the case of LDH, where even in the presence of 50 μM melatonin at the same nitric oxide concentration activity of the enzyme was unaffected (Fig. 4). The observed prooxidative rather than antioxidative effect of melatonin in studied conditions may be in part due to interactions between products of reaction of NO with melatonin and GAPDH. As found by Turjanski et al. [58] the main product of the reaction between nitric oxide and melatonin is N^1-nitrosomelatonin (Mel-NO) according to Eq. (10):

$$4\,NO + O_2 + 2\,Mel \rightarrow 2\,Mel - NO + 2\,HNO_2 \quad (10)$$

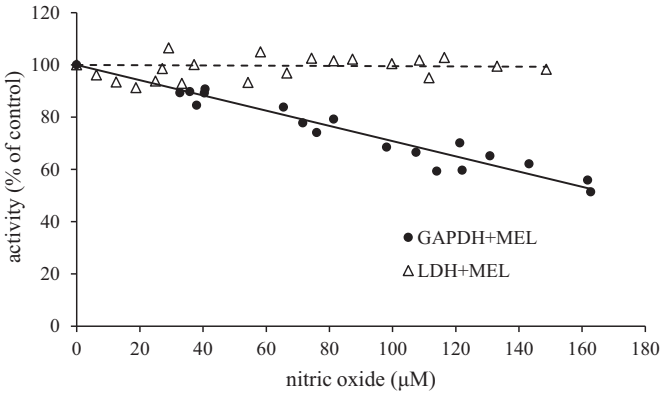

Fig. 4. The influence of melatonin on the nitric oxide induced functional changes of GAPDH and LDH. Proteins were incubated for 60 min prior to activity assay with the increasing concentration of nitric oxide and in the presence of 50 μM melatonin. Data are expressed as a percent of control values.

The mechanism of nitric oxide based melatonin nitrosation is either radical or N_2O_3 dependent [55]. Nascent N^1-nitrosomelatonin is regarded as a potent transnitrosylating agent that may block GAPDH activity at quite low concentrations [59,60]. That may also explain increased inactivation of GAPDH caused by nitric oxide and its oxidation products in the presence of melatonin. In the performed studies melatonin present in protein samples might interact with NO and its oxidation products to give N^1-nitrosomelatonin which in turn could transnitrosylate GAPDH in the course of incubation.

3.5. The influence of melatonin on the amount of reduced thiols in the enzymes exposed to nitric oxide

Analysis of the amount of unmodified thiols (Fig. 5) in GAPDH shows that increased inactivation of the enzyme in the presence of both nitric oxide and melatonin is mainly due to further decline of protein free thiols. The presence of melatonin in studied system was responsible for about 15% decrease in the number of free thiols under the applied conditions (Figs. 2, 5). Contrary to GAPDH, LDH thiols were not affected by the presence of melatonin (Figs. 2, 5).

3.6. The influence of resveratrol on the function of GAPDH and LDH in the presence of nitric oxide and its secondary products of oxidation

Several data indicate that resveratrol is a good scavenger of RNS such as NO, NO_2, $ONOO^-$ [61–63]. The main secondary scavenging

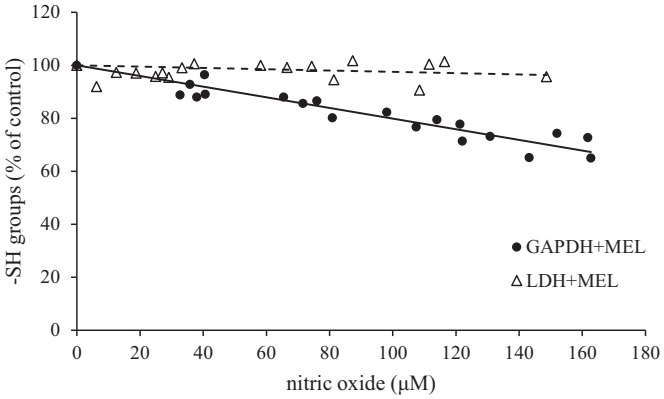

Fig. 5. The number of reduced thiol groups in GAPDH and LDH exposed to different concentrations of NO in the presence of 50 μM melatonin. After 60 min incubation of protein samples free thiol number was assessed spectrophotometrically by measuring the absorbance of TNB^{2-} anion at 412 nm and expressed as percent of control values.

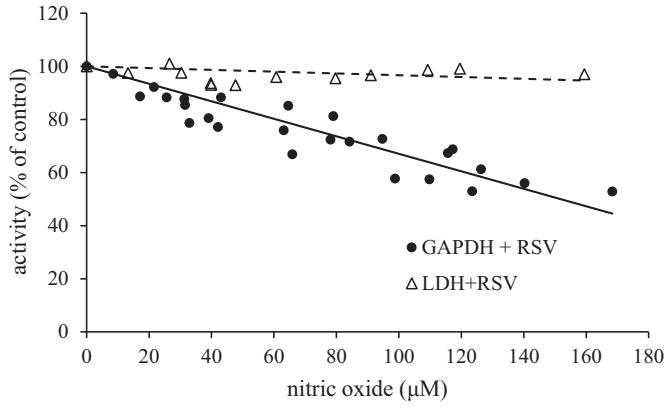

Fig. 6. Activity assay of GAPDH and LDH exposed to different concentrations of nitric oxide in the presence of 50 μM resveratrol. Prior to proteins activity determination, samples were incubated for 60 min in the dark. Data are expressed as a percent of control values.

product of both NO and NO_2 is phenoxyl radical. Hence, increased inactivation of GAPDH exposed to nitric oxide in the presence of resveratrol may be caused by the interaction of generated resveratrol radicals with thiol groups of the active site of GAPDH (Figs. 1, 6). Proposed mechanism is consistent with our previously published data where resveratrol radicals formed in the presence of superoxide anion radical were responsible for increased inactivation of GAPDH in comparison to superoxide anion radical alone [42,64].

3.7. The number of free thiols in the dehydrogenases exposed to nitric oxide in the presence of resveratrol

As shown in Figs. 6 and 7 the increased inactivation of GAPDH caused by nitric oxide in the presence of resveratrol and nascent phenoxyl radicals of resveratrol does not correlate with the increased loss of –SH groups in the protein. Contrary to GAPDH the number of free sulfhydryls in the LDH molecule is almost unaffected in the presence of both nitric oxide and resveratrol (Fig. 7). The obtained results are consistent with overall insensitivity of LDH molecule to functional changes upon its interaction with NO both alone and in the presence of used antioxidants.

3.8. Binding of bis-ANS fluorescence probe to proteins exposed to nitric oxide in the presence and absence of the antioxidants

Fluorescence dyes such as bis-ANS are well known for their ability

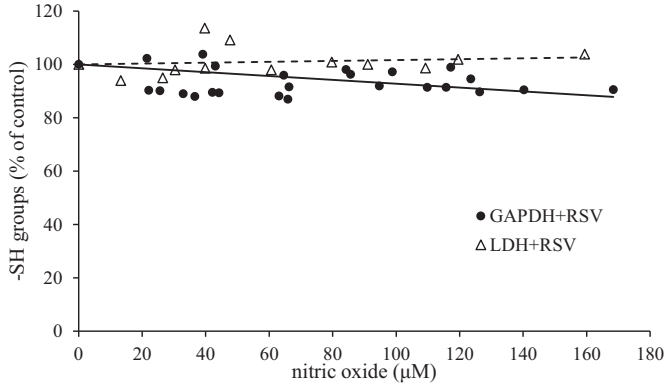

Fig. 7. The number of reduced thiol groups in GAPDH and LDH exposed to different concentrations of NO in the presence of 50 μM resveratrol. After 60 min incubation of protein samples free thiol number was assessed spectrophotometrically by measuring the absorbance of TNB^{2-} anion at 412 nm and expressed as a percent of control values.

Table 2
Relative changes of bis-ANS fluorescence upon its binding to studied intact proteins and proteins treated with NO both in the absence and in the presence of resveratrol or melatonin, respectively. The fluorescence data are expressed as a percent of control (intact proteins) values.

Protein	C_{NO} [μM]	Relative fluorescence values [%]			
		Intact protein	Protein + RSV	Protein + NO	Protein + RSV + NO
LDH	159.4	100.0	79.5	88.1	61.4
GAPDH	143.1	100.0	94.5	85.4	49.1
			Protein + MEL	Protein + NO	Protein + MEL + NO
LDH	148.7	100.0	88.5	87.5	63.5
GAPDH	152.0	100.0	97.3	88.8	74.9

to probe conformational changes of proteins [65]. Hydrophobic and to a lesser extent electrostatic interactions are the main interactions that enhance intrinsic fluorescence of bis-ANS upon its interaction with protein. As displayed in Table 2, exposing both dehydrogenases to NO at a concentration of ~150 μM results in similar decrease of bis-ANS fluorescence (~13%). The presence of antioxidant and nitric oxide in the studied system caused further decrease in the dye fluorescence. Both antioxidants in the presence of NO caused similar conformational changes of LDH resulting in about 40% decrease of bis-ANS fluorescence. In comparison to LDH, GAPDH is far more prone to conformational changes in the presence of NO and resveratrol resulting in the decrease of bis-ANS binding (50% compared to intact protein). The results of GAPDH structural changes induced by NO in the presence of resveratrol correlate with increased inactivation of GAPDH in the presence of NO and RSV (Fig. 6). Formed in the reaction with NO or other one electron oxidants phenoxyl radicals of resveratrol are more damaging to the structure of GAPDH as it was verified in our previous study [64]. Smaller changes of bis-ANS fluorescence were observed in the case of GAPDH exposed to NO in the presence of melatonin (25%) (Table 2) but again the structural changes correlate with increased inactivation of GAPDH caused by NO in the presence of this antioxidant (Fig. 4). Increased inactivation and structural changes of GAPDH in the presence of both used antioxidants confirm their role as prooxidants but not antioxidants in studied system. Furthermore, augmented changes of GAPDH structure caused by NO in the presence of resveratrol and melatonin may exclude physiological role of NO signaling and lead even to GAPDH aggregation [21,22].

It is necessary to take into account that resveratrol and melatonin are hydrophobic compounds that may interact with protein hydrophobic sites causing the reduction of bis-ANS fluorescence compared to intact protein (Table 2). However, the effect of nitric oxide in the presence of both antioxidants on the reduction of bis-ANS fluorescence is always higher than the sum of such effects of antioxidants and NO alone (Table 2).

3.9. Prediction of potential S-nitrosylation sites in the studied dehydrogenases

Computational prediction tools of S-nitrosylation sites in proteins are robust, competitive and complementary to experimental methods [66]. Here, we used the GPS-SNO [45] method to predict potential S-nitrosylation sites in the studied enzymes: GAPDH (P46406) and LDH (P13491). According to this prediction rabbit GAPDH may be modified at three such sites (Cys-149, Cys-153, Cys-244) whereas, LDH contain only one cysteine that is prone to NO modifications (Cys-162) (Fig. 8). Predicted results are consistent with existing data for dehydrogenases of other species. According to online database for cysteine S-nitrosylation (db-SNO) [66] human GAPDH contain three S-nitrosylation sites (Cys-151, Cys-246, Cys-155) that has been experimentally verified and rat

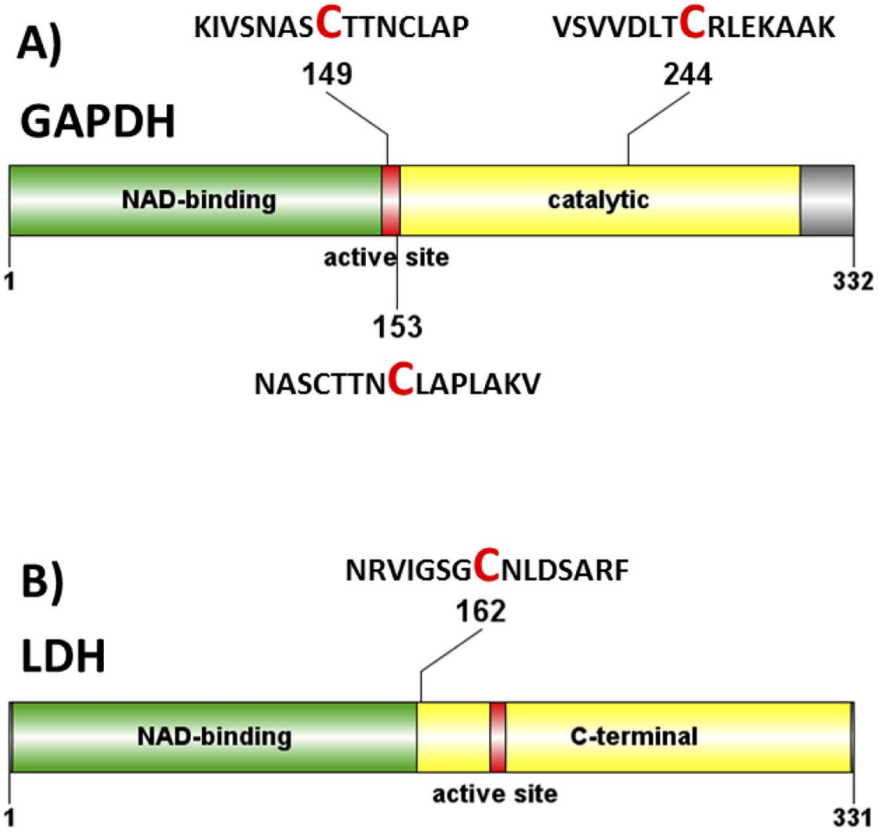

Fig. 8. GPS-SNO predicted S-nitrosylated sites in rabbit GAPDH (A) (Uniprot No P46406) and rabbit LDH (B) (Uniprot No P13491). Modified cysteine residues are marked in red with the flanking amino acids in black. Medium threshold was used as a default.

LDH has one such site (Cys-162) [67].

4. Conclusions

Nitric oxide and its oxidation products inactivate GAPDH in a dose dependent manner by modifying its catalytic cysteine residues. This modification can be reversed by the action of sulfhydryl reducing agents (GSH, DTT). In comparison with GAPDH, NO even at relatively high concentrations is unable to influence the function of LDH as a pyruvate to lactate converting enzyme. The enzymatic activity of LDH does not rely on the presence of cysteine residues in its active center which additionally confirms role of NO as a cysteine modifying molecule. Under the applied conditions the antioxidants used (melatonin and resveratrol) increased deleterious effect of RNS on GAPDH function and potentiated structural modifications of GAPDH and LDH. Our results indicate that, though via different mechanisms, both resveratrol and melatonin displayed prooxidative properties in the system examined. The most likely resveratrol and melatonin derived species that enhance functional and structural protein modifications in the studied conditions are phenoxyl radicals and nitrosomelatonin respectively.

Conflict of interest

None.

Transparency document

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

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Appendix A. Statistical analysis

The results of at least 3 independent experiments ($n \geq 3$) of activity and –SH groups amount changes of GAPDH and LDH exposed to NO in the presence and absence of resveratrol and melatonin were fitted to linear regression model using the least squares method. The differences between mean slopes $\pm$ SD of the activity or SH- groups number between GAPDH and LDH in the presence and absence of used antioxidants were further analyzed using two-way ANOVA and post hoc Scheffe's (Tables A.1, A.2). All statistical analysis was performed using Statistica (Stat-Soft Poland) software.

Table A.1
Parameters *a*, linear regression slopes ± SD of relative activity data in the function of nitric oxide concentration in the studied proteins systems. Statistically significant differences were verified between activity of both proteins GAPDH and LDH treated with NO and between GAPDH treated with NO and GAPDH treated with NO in the presence of both antioxidants (melatonin and resveratrol). Post hoc Scheffe's test, $p < 0.005$.

Studied protein systems	GAPDH + NO	GAPDH + MEL + NO	GAPDH + RSV + NO	LDH + NO	LDH + MEL + NO	LDH + RSV + NO
Parameter *a* ± SD of relative activity	− 0.148 ± 0.02	− 0.283 ± 0.018	− 0.280 ± 0.024	− 0.0161 ± 0.011	0.030 ± 0.021	0.00061 ± 0.017

Table A.2
Parameters *a*, linear regression slopes ± SD of relative –SH group amount in the function of nitric oxide concentration in the studied proteins systems. Using post hoc Scheffe's test no statistically significant differences were confirmed between relative –SH groups amount of GAPDH (LDH) treated with NO and GAPDH (LDH) treated with NO in the presence of antioxidants. Similarly, no statistically significant differences were observed between –SH groups amount of GAPDH and LDH treated with NO in the absence of melatonin and resveratrol.

Studied protein systems	GAPDH + NO	GAPDH + MEL + NO	GAPDH + RSV + NO	LDH + NO	LDH + MEL + NO	LDH + RSV + NO
Parameter *a* ± SD of relative –SH groups amount	− 0.099 ± 0.019	− 0.189 ± 0.016	− 0.0181 ± 0.023	− 0.0344 ± 0.012	0.0053 ± 0.018	0.0218 ± 0.035

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Analysis of Potential Binding Sites of 3,5,4′-Trihydroxystilbene (Resveratrol) and *trans*-3,3′,5,5′-Tetrahydroxy-4′-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols

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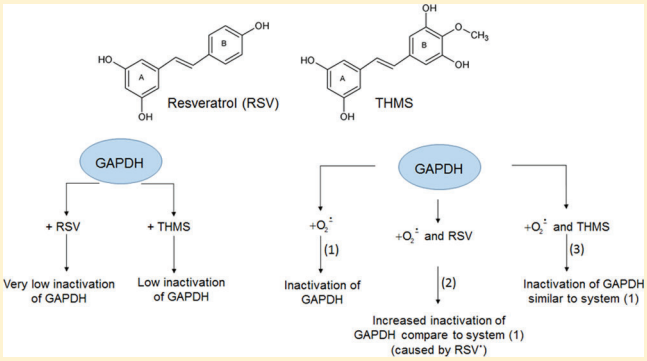
ABSTRACT: The presented study analyzed potential binding sites of 3,5,4′-trihydroxystilbene (resveratrol, RSV) and its derivative, *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (THMS) to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The effects of stilbene analogs on the structure of GAPDH were determined by fluorescence spectroscopy and ζ potential measurements. To what extent the studied compounds affect the activity of the enzyme was also assessed. A computational ligand-docking study showed that there are 11 potential binding sites of RSV and 8 such sites of THMS in the GAPDH molecule. While resveratrol does not significantly affect the activity of the dehydrogenase upon binding to it, THMS leads to approximately 10% inactivation of this enzyme. THMS has no effect on GAPDH inactivation induced by the superoxide anion radical, in contrast to resveratrol, which increases dehydrogenase inactivation.



INTRODUCTION

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a protein ubiquitously expressed in all organisms studied to date. It is abundant in almost all cells and is often used as a housekeeping protein.[1,2] Under normal conditions, GAPDH is located mainly in the cytoplasm. Mammalian GAPDH is a tetramer that consists of four equal, approximately 36 kDa monomers. The main function of GAPDH is the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in the presence of the cofactor NAD^+. In addition to its catalytic, enzymatic function, GAPDH is a classic example of multifunctional proteins, also known as moonlighting proteins.[3] The multifunctional properties of GAPDH are linked to its oligomeric status, redox modifications of the catalytic cysteine residues, and the cellular localization of the protein.[4,5] GAPDH is a promising therapeutic target for several diseases because of its involvement in such cellular processes as DNA repair, transcription activation, vesicle shuttling, membrane fusion, and nuclear RNA transport, to name just a few.[6−10] Under conditions of oxidative stress, the S-nitrosylation of cysteine residues in GAPDH leads to its binding to ubiquitin ligase Siah1.[11] The translocation of this protein complex to the nucleus is responsible for the initiation of the cellular death cascade and apoptosis.[12,13] Recent data suggest that oxidatively modified GAPDH may initiate apoptotic cell death in neuronal cells.[14−16] Moreover, there are numerous studies indicating that oxidatively modified GAPDH is one of the main components of extra- and intracellular amyloid lesions in such neurodegenerative disorders as Alzheimer's and Parkinson's diseases.[8,17−22]

Some of the most potent antioxidants are polyphenols, such as resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) and its derivatives. They are naturally occurring phytoalexins with wide-ranging bioactivity exhibiting cardioprotective, neuroprotective, anti-inflammatory, anticancer, and antiaging properties.[23−27] Natural polyphenols are one of the most active amyloid inhibitors, and resveratrol has recently been reported to inhibit and remodel the human islet amyloid polypeptide oligomers and fibrils.[28−31]

Along with resveratrol, this study also investigated its derivative, *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene (THMS). This compound is found, among others, in the

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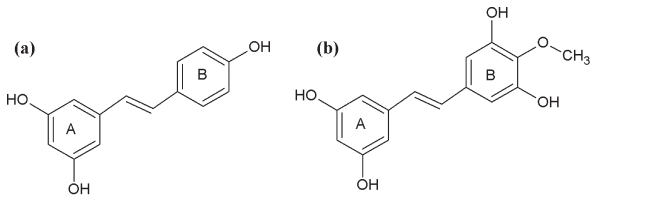
skin of peanuts and the bark of the shrub *Yucca schidigera* (Scheme 1).[32,33]

Scheme 1. Chemical Structures of Resveratrol (a) and THMS (b)



resveratrol

trans-3,3',5,5'-tetrahydroxy-4-methoxystilbene (THMS)

Numerous structure−activity relationship studies have revealed that the 4′-OH group in the phenol ring of resveratrol is responsible for its antioxidant and biological activity.[34−36] The free-radical scavenging mechanism utilized by resveratrol involves the generation of 4′-phenoxy radicals followed by semiquinone and quinone structures. Generally regarded as a good antioxidant, resveratrol may also exhibit pro-oxidant properties toward proteins and DNA.[37−39] In our previous work, we presented results indicating the pro-oxidant properties of resveratrol toward GAPDH in the examined system in vitro.[37]

The presented study investigated the effects of resveratrol and THMS on glyceraldehyde 3-phosphate dehydrogenase. Computational molecular docking was used to identify and characterize the potential binding sites of the polyphenols to the dehydrogenase. Furthermore, the study examined in what ways the binding of the polyphenols to GAPDH affects the structure and function of the latter in the presence and absence of the superoxide anion radical.

MATERIALS AND METHODS

Materials. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from rabbit muscle, *trans*-3,4′,5-trihydroxystilbene (resveratrol, RSV), and all other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated. *trans*-3,3′,5,5′-Tetrahydroxy-4′-methoxystilbene (THMS) was isolated from *Yucca schidigera* bark as previously described.[32]

Preparation of Protein and Polyphenol Solutions. Protein solution was prepared by dissolving GAPDH in 0.1 M sodium phosphate buffer at pH 7.4. Protein concentration was determined spectrophotometrically at 280 nm, using the extinction coefficient $E^{1\%} = 10$ (GAPDH, MW = 143 000). Resveratrol stock solution was prepared in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. After extraction and separation, THMS was provided as a stock solution at a concentration of 25 mg/mL in a mixture of DMSO and water 50:50 (v:v). All stock solutions of phenolic compounds were prepared and stored frozen at −20 °C in the dark to avoid isomerization of the compounds. For each experiment, a fresh dilution of each compound was prepared in 0.1 M sodium phosphate buffer (pH 7.4), kept in the dark, and used only for a single experiment.

Assay of GAPDH Activity in the Presence of Phenolic Compounds and Reactive Oxygen Species. The activity of the enzyme at a concentration of 1.4 μM was determined spectrophotometrically by measuring the rate of reduction of NAD^+ to NADH at 340 nm in a reaction catalyzed by GAPDH as described previously.[40] The relative activity of GAPDH as a percentage of the control value was determined either in the presence of 50 μM of each polyphenolic compound alone or additionally with the superoxide anion radical (concentration range 0−80 μM). The superoxide anion radical was generated by 55 min incubation of 0.5 mM/mL xanthine with xanthine oxidase 0.005 U/mL in the presence of catalase 330 U/mL. The concentration of the generated superoxide anion was determined spectrophotometrically in a reaction with cytochrome *c* (0.2 mM) and the excess of catalase (660 U/mL) as described previously.[37] All measurements were carried out at 24 ± 1 °C using a CARY-1 apparatus (Varian, Melbourne, Australia).

Fluorescence Quenching. Fluorescence quenching was analyzed to investigate polyphenol−GAPDH interactions. Fluorimetric experiments were carried out on a Varian Cary Eclipse fluorimeter in a thermostatic cuvette at 298 K (25 °C). GAPDH concentration was constant at 1.4 μM in 0.1 M phosphate buffer (pH 7.4) and the concentration of the phenolic compounds varied from 0.25 to 30 μM in the cuvette. Fluorescence spectra were recorded at λ_{exc} = 280 nm and at λ_{em} from 300 to 420 nm. The excitation and emission slits were 5 nm wide each. Corrected fluorescence intensity at maximum emission was used to calculate the Stern−Volmer quenching constants.[41] All experiments were done at least in triplicate.

Fluorescence correction was carried out according to the procedure described by Birdsall et al. (1983), involving both the absorption of light excited by the ligands (the studied polyphenols) and the absorption of emitted light.[42] Corrected fluorescence intensities for the various wavelengths were calculated according to the following equation:

$$F_{corr} = C_1 C_2 F_{obs} \quad (1)$$

C_1 is the correction factor for excitation light determined using the formula

$$C_1 = 10^{(A)(0.5)} \quad (2)$$

where A is the absorbance of excitation light at a wavelength of 280 nm and 0.5 represents half of the cuvette length along the excitation direction. C_2 is the correction factor for emitted light determined using the formula

$$C_2 = \frac{2.303(A)(0.4)}{1 - 10^{-A(0.4)}} \quad (3)$$

where A is the absorbance of particular wavelengths of light emitted in the range of 320−400 nm at a given polyphenol concentration and the value 0.4 represents cuvette width with respect to the emission direction.

Fluorescence Lifetime Measurements for GAPDH. Fluorescence lifetime measurements were carried out at 22 °C with a FL900CDT time-correlated single photon counting fluorimeter from Edinburgh Analytical Instruments. The excitation and emission wavelengths were set to 295 and 340 nm, respectively. Data acquisition and analysis were performed using the software provided by Edinburgh Analytical Instruments. Measurements were conducted at the Institute of General Food Chemistry, University of Technology, Lodz.

ζ Potential Measurements. The ζ potential measurements were performed using a Zetasizer Nano ZS from Malvern, which employs electrophoretic light scattering techniques. This enabled measurement of the electrophoretic

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mobility of molecules in the solution. The ζ potential was calculated directly from the Helmholtz−Smoluchowski equation using the Malvern software. Measurements of the potential were performed at 37 °C with three repetitions. Increasing concentrations of polyphenols (0.25−25 μM) were added to 1.4 μM GAPDH, and the ζ potential was measured.

Molecular Modeling and Docking. The 3D structures of resveratrol and THMS were generated by the DFT method using CAChe WorkSystem Pro software (Fujitsu Ltd.). Docking studies were conducted using a Discovery Studio molecular simulation system with the LigandFit and CDOCKER algorithms (DS, Accelrys Inc.). Molecular graphics were produced using the UCSF Chimera package.[43] The GAPDH structure used in this work was from Protein Data Bank, obtained by X-ray diffraction (PDB code 1J0X).[44] The four monomers forming the tetramer are not 3D identical. Ligand poses are evaluated and prioritized according to the value of the dock-score function (D-S).

Statistical Analysis. All results are expressed as mean ± SD of at least three independent experiments. Statistical significance was evaluated by means of Student's *t* test using Statistica software. Results were deemed to be statistically significantly different at $*p < 0.01$, $**p < 0.001$.

RESULTS AND DISCUSSION

Analysis of Possible Resveratrol and THMS Binding Sites to GAPDH by Means of Molecular Docking. Plausible binding sites of two ligands (resveratrol and THMS) to the GAPDH molecule were analyzed. The structure of the studied polyphenols is shown in Scheme 1, and their basic structural and physicochemical properties are given in Table 1. The studied compounds are similar in that both contain a stilbene backbone. However, they slightly differ in size and have different physicochemical properties. Resveratrol has three hydroxyl groups (at positions 3, 5, and 4′), while THMS has four hydroxyl groups (at positions 3, 3′, 5, 5′) and additionally a methoxy group in the B ring at position 4′, so it is the larger molecule. Resveratrol has a molecular weight of 228.2 Da and a molecular volume of 177.67 Å^3, while the corresponding figures for THMS are 274.27 Da and 217.11 Å^3. Furthermore, the compounds differ in their polarity and dipole moment.

Table 1. Physicochemical Properties of Resveratrol and THMS

parameter	resveratrol	THMS
molecular weight	228.24	274.27
molecular volume (Å^3)	177.67	217.11
dipole magnitude (D)	0.757	1.14
molecular surface area (Å^2)	229.51	276.17
fraction polar area (%)	26.4	32.6
molecular solubility	−3.226	−2.686

Analysis conducted using the Ligand Fit algorithm detected 48 cavities on the molecular surface of GAPDH that could potentially harbor the studied polyphenols. The molecular docking method revealed 11 most probable binding sites of resveratrol to the GAPDH tetramer with dock-score (D-S) values ranging from 20.5 to 48.0. In turn, eight such sites were found for THMS, with D-S values from 36.1 to 47.7. More detailed analysis of ligand locations in the GAPDH molecule was conducted for two cases:

(1) the region with the highest accessible volume, which is located where the subunits come in contact.
(2) the region near the active site (near Cys-149).

Analysis also included

(3) the location of Trp residues in the neighborhood of the ligand-binding sites.

1. Analysis of the Region with the Greatest Available Volume in the GAPDH Tetramer. The largest accessible region in GAPDH is located inside the molecule, where the subunits come into contact. That region widens toward the surface of the tetramer along the O−Q interface on one side and the P−R interface on the other side. In this region, 10 ligand binding positions with the highest dock-scores (D-S) were analyzed. In the case of resveratrol, the highest D-S values (46.0−48.0) were found for the site located at the interface between the P and R subunits (Figure 1).

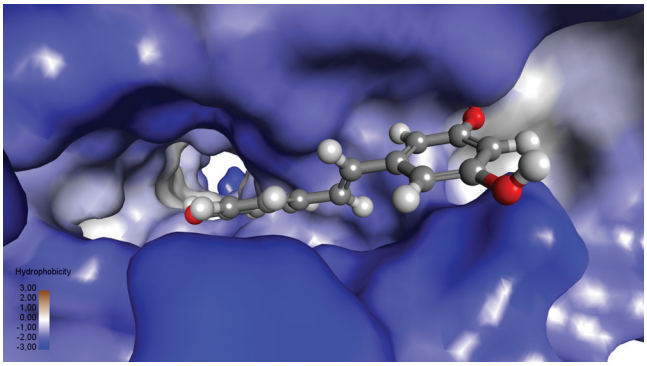

Figure 1. The largest accessible region in GAPDH for resveratrol binding is located inside the molecule, where the subunits come into contact.

Analysis of interactions shows that the optimum resveratrol poses are stabilized by hydrophobic and electrostatic interactions between Lys 52 (R) and the stilbene ring and by a hydrogen bond with Asp 286 (P). In the case of THMS, the highest D-S values (44.6−46.4) were found for the location between the subunits O and Q. Analysis of interactions shows that the identified optimum poses are stabilized by hydrogen bonds with Cys 281 (O) and Asn 284 (Q), as well as Ser 48 (O) (only the location with the highest D-S). Differences between resveratrol and THMS in terms of locations with the greatest accessible volume are probably attributable to the asymmetrical 1J0X structure described in the PDB database.

2. Analysis of Ligand Locations within the Active Site. Analysis was conducted using the CDOCKER protocol for the active site of the subunit O.[45] Interactions were characterized by -CDOCKER INTERACTION ENERGY (-CD-Int-E) values, which describe the overall energy of the ligand−protein interaction (the higher the value, the higher is the interaction energy). The differences in the CDOCKER INTERACTION ENERGY between the studied ligands (as shown in Table 2) are related to the number of amino acid residues with which the ligands can interact. Thus, the differences are determined by the structure of the ligands and especially by their ability to form hydrogen bonds (Table 2). Analysis revealed that the studied ligands may be located within the active site. The -CD-Int-E values for RSV ranged from 26.1 to 29.9. At that site, RSV binding is mostly stabilized by hydrogen bonds with Arg 231 and Ser 119 and by hydrophobic interactions with His 176 and

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Table 2. Most Frequently Occurring Interactions of Resveratrol and THMS at the Active Site of the O Subunit of GAPDH[a]

ligand	-CDOCKER INTERACTION ENERGY	hydrogen bonds (H-bonds)	hydrophobic interactions
resveratrol	26.14−29.89	O:Arg231 (8), O:Ser119 (6)	O:His176 (7), Tyr317 (2, pose with the highest D-S)
THMS	30.07−35.98	O:Glu314 (8), O:Ala177 (5), Ile178 (5), O:Asn313 (4), O:Thr208 (4), O:Cys149 (1, pose with the highest D-S)	O:Thr179 (5)

[a]Number in parentheses represents how many times the residue was involved in interaction/10 best poses.

Tyr 317 (Figure 2A and Table 2). It should be noted that no direct interaction between the ligand and Cys 149 was identified.

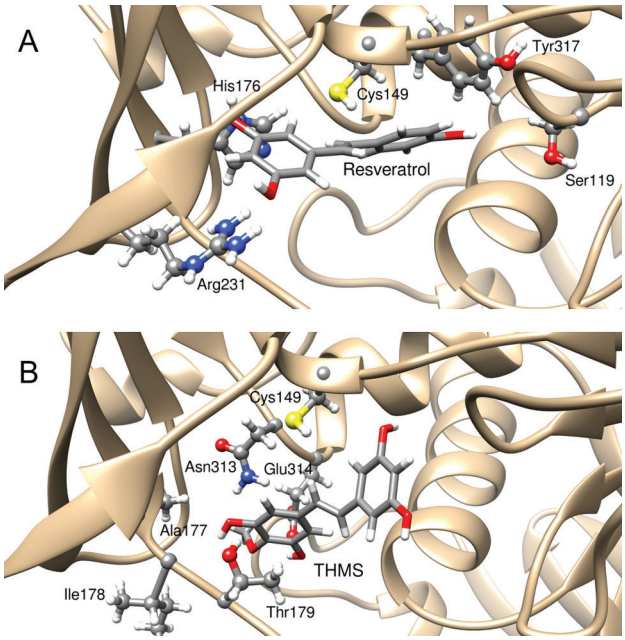

Figure 2. Localization of resveratrol (A) and THMS (B) within the active site of GAPDH.

In the case of THMS, the -CD-Int-E values are 30.1−36.0. THMS binding at this site is stabilized by a much greater number of hydrogen bonds than in the case of resveratrol; these H-bonds involve the following residues Glu 314, Ala 177, Ile 178, Asn 313, and Thr 208, as well as the amide group of Cys 149 (only for the pose with the highest score). Additionally, there is also a hydrophobic interaction with Thr 179 (Table 2 and Figure 2B).

3. Analysis of the Location of Tryptophan Residues near the Ligand Binding Sites. Analysis showed the presence of tryptophan residues near all the identified potential binding sites of the studied compounds. There were two to four tryptophan residues close to every ligand binding site, within less than 20 Å from the ligand. As it was already mentioned, 11 possible binding sites of resveratrol in the GAPDH tetramer were identified. In all cases, Trp 193 was found less than 20 Å from the RSV binding site. Moreover, Trp 84 was detected near eight of those sites. It should be noted that in three cases that tryptophan was located within 10 Å from RSV (Figure 3). Trp 193 resides on the surface of the protein, while Trp 84 is situated just under the surface of the molecule.

Similar to RSV, tryptophan residues were found near all eight identified THMS binding sites: in all cases they were Trp 193 residues located within 20 Å from the ligand. Additionally, Trp

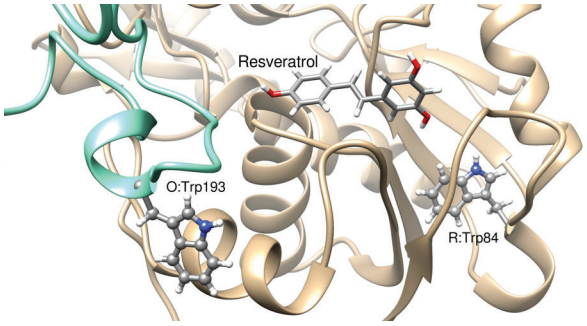

Figure 3. Positions of tryptophan residues (Trp 84 and Trp 193) in GAPDH molecule near potential binding sites of the resveratrol.

84 was identified near five of those sites (in three cases it was situated less than 10 Å from the ligand). The tryptophan residue found least often near the binding sites of both ligands was Trp 310.

Effect of the Studied Polyphenols on the Activity of GAPDH. In the next stage of the study, it was investigated whether and to what extent the studied polyphenols influenced the functional properties of GAPDH. The enzyme (at a concentration of 1.4 μM) was incubated with the polyphenols (50 μM) at room temperature for 55 min. Under the above incubation conditions, resveratrol did not affect GAPDH activity to a significant extent. However, the activity of the enzyme declined under the influence of THMS (Figure 4). GAPDH became slightly inactivated already after 2 min of incubation, while after 25 min its activity decreased by 14%. It seems likely that this effect of THMS is attributable to interactions of that ligand within the active site of the dehydrogenase. As it was shown in section 2, at that site

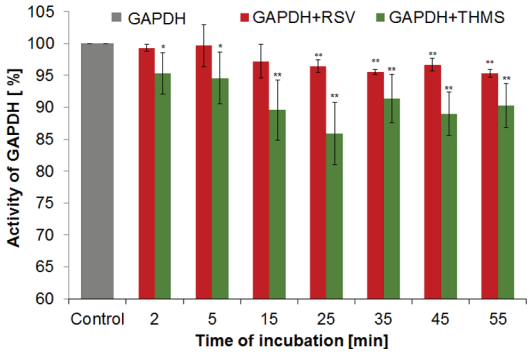

Figure 4. Effects of resveratrol and THMS on the activity of GAPDH as measured during 55 min of incubation of 1.4 μM GAPDH with 50 μM polyphenols. Values are expressed as percentages referring to the activity of untreated GAPDH (control). Data are the mean ± SD of four to six independent measurements. Asterisks indicate significant differences between the activity of the control and GAPDH treated with polyphenols: (*) $p < 0.01$, (**) $p < 0.001$ (t test).

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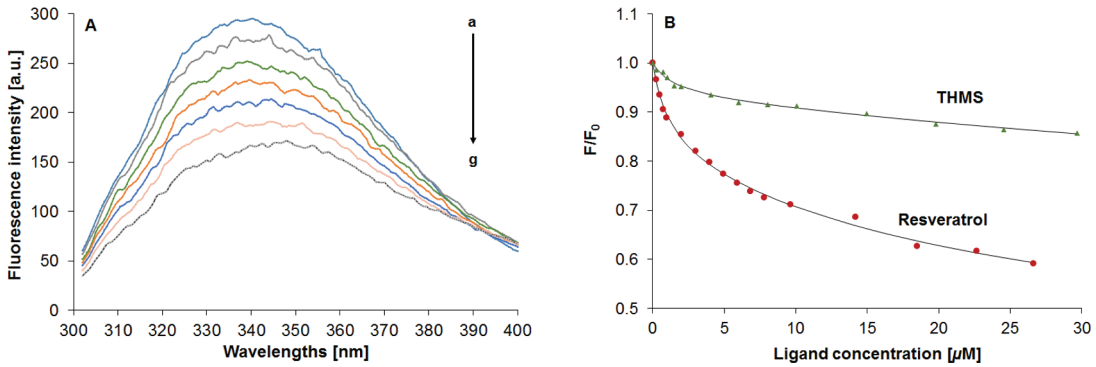

Figure 5. (A) Example of a fluorescence emission spectrum of the RSV–GAPDH system in 0.1 M phosphate buffer at pH 7.4 for free GAPDH (a) and GAPDH with RSV (b–g) at concentrations of 0.5, 2, 3.95, 6.84, 14.16, and 18.48 μM. (B) Quenching of GAPDH fluorescence by RSV and THMS; dots represent experimental values, while the continuous line represents values calculated from fitting with eq 4.

Table 3. Stern–Volmer Constants for Quenching of GAPDH Fluorescence Calculated from the Fitting of Experimental Data with Equation 2[a]

polyphenol	K_{SV1} (μM^{-1})	f_1	K_{SV2} (μM^{-1})	f_2	f_0
resveratrol	0.705 ± 0.214	0.189 ± 0.012	0.0286 ± 0.0078	0.439 ± 0.028	0.375 ± 0.041
THMS	0.404 ± 0.323	0.070 ± 0.006	0.0049 ± 0.0010	0.530 ± 0.006	0.400 ± 0.050

[a]The Stern–Volmer quenching constant values (K_{SV1} and K_{SV2}) are averages of three replicate runs for polyphenol–GAPDH system.

THMS is bound much more strongly than RSV, as it is stabilized by a greater number of hydrogen bonds (two for RSV vs six for THMS, including one formed directly with the amide group of Cys 149; see Table 2). Therefore, it may be assumed that it is much more difficult for the substrate to displace THMS than RSV.

Quenching of GAPDH Fluorescence by the Polyphenols. Another line of evidence for interactions between the studied polyphenols and GAPDH is the fact that these compounds quench GAPDH fluorescence (which is mainly attributable to tryptophan residues). The excitation wavelength of 280 nm was selected because of the fact that RSV and THMS exhibit strong absorption at 290–295 nm. An excitation wavelength of 295 nm, which is best suited for tryptophan residues in proteins, would considerably complicate measurements and interpretation of results. According to literature data, at an excitation wavelength of 280 nm, fluorescence observed at an emission wavelength of 340 nm comes mostly from tryptophan residues and, to a much smaller degree, from tyrosine residues.[41] Thus, the obtained results primarily represent changes in fluorescence attributable to tryptophan residues. Figure 5A shows emission spectra of GAPDH in the presence of resveratrol.

In the absence of the ligand, the emission maximum occurs at approximately 339.5 nm. With increasing resveratrol concentrations, the intensity of fluorescence decreases, with a slight shift of the emission maximum toward longer wavelengths (to approximately 344 nm at the highest concentration of resveratrol). GAPDH fluorescence was quenched by THMS to a much smaller extent, without a shift in the emission maximum. The Stern–Volmer plot (the relationship between F_0/F and quencher concentration) was nonlinear. At low concentrations of the polyphenols, fluorescence was quenched with greater efficiency (Figure 5B and Table 3). The relationship between relative fluorescence intensity and quencher (polyphenol) concentration may be satisfactorily described with the following equation:

$$\frac{F}{F_0} = f_0 + \sum_{i=1}^{2} \frac{f_i}{1 + K_{SV_i}[Q]} \quad (4)$$

where F_0 is GAPDH fluorescence intensity in the absence of a quencher (polyphenol), F is fluorescence intensity in the presence of polyphenol, f_0 is fluorescence component that was not quenched, f_i is the ith component of the total fluorescence of the system (i from 1 to 2), K_{SV_i} is the Stern–Volmer quenching constant for the ith component, $[Q]$ is the concentration of the quencher (polyphenol).

The data presented in Table 3 describe the two-phase nature of the fluorescence quenching process and differences in the efficiency of quenching by the studied polyphenols. The values f_1 and f_2 represent the fluorescence components quenched with high and low efficiency at constants K_{SV1} and K_{SV2}, respectively. Component f_0 represents nonquenched fluorescence. The low quenching constant K_{SV2} of the prevalent fluorescence component f_2 is approximately 25 and 82 times lower than constant K_{SV1} for resveratrol and THMS, respectively.

Furthermore, the above analysis of binding sites of the polyphenols to GAPDH indicates that Trp 193, exposed on the molecule surface, may be the first residue to be quenched. This tryptophan is probably quenched with high efficiency, corresponding to K_{SV1}. Trp 193 was found near all 11 binding sites of resveratrol and all 8 binding sites of THMS, within 20 Å of the polyphenols. Trp 84, located close to the protein surface but still deeper in the molecular structure, is probably quenched with a much lower efficiency, corresponding to K_{SV2}. Trp 84 was found near eight binding sites of RSV and five binding sites of THMS. In turn, the quenching of Trp 310 seems unlikely, as it is located deep inside the tetramer molecule. Analysis of binding sites showed that the studied polyphenols are least likely to be located near the Trp 310 residue. Unquenched fluorescence ($f_0 = 0.4$) comes primarily from Trp 310, which is embedded deep in the molecule and, to a much smaller extent, from tyrosine residues. For tryptophan residues, the quantum efficiency of fluorescence in a hydrophobic environment is much higher than in a hydrophilic one.[46] Thus, the fact that the

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Table 4. Lifetimes of GAPDH Tryptophan Fluorescence in the Absence and Presence of Polyphenols

system	polyphenol [μM]	τ_1 (ns)	f_1	τ_2 (ns)	f_2	χ^2
GAPDH	0	1.35 ± 0.02	0.677	3.22 ± 0.04	0.323	1.837
GAPDH + RSV	5	1.36 ± 0.02	0.700	3.26 ± 0.04	0.300	1.763
	25	1.39 ± 0.01	0.746	3.53 ± 0.05	0.254	1.731
GAPDH + THMS	5	1.33 ± 0.02	0.707	3.28 ± 0.04	0.293	1.783
	25	1.32 ± 0.02	0.722	3.22 ± 0.05	0.278	1.965

fluorescence components f_2 and f_0 are much greater than f_1 is probably caused by the higher quantum efficiency of the fluorescence of Trp 310 and Trp 84 (which are located in a hydrophobic environment) as compared to Trp 193, which is exposed on the molecule surface. As can be seen from Table 3 and the corresponding figure, the efficiency of fluorescence quenching by THMS is much lower than that by RSV. The quenching constants K_{SV1} and K_{SV2} are approximately 1.8 and 6 times higher for RSV, respectively. This reflects the number of identified probable binding sites (11 for RSV and 8 for THMS). The lower number of binding sites and the much lower quenching efficiency of THMS is attributable to the presence of a methoxy group in the molecule, which in some cases may prevent THMS from binding to the protein, hindering the formation of hydrogen bonds involving the hydroxyl groups at positions 3′ and 5′.

Fluorescence Lifetime. Tryptophan fluorescence lifetime in the absence and presence of the ligands was measured in order to determine the mechanism of fluorescence quenching (Table 4). Fluorescence decay may be satisfactorily described by the following biexponential equation:

$$F = A_0 + B_1 e^{-t/\tau_1} + B_2 e^{-t/\tau_2} \quad (5)$$

In the absence of polyphenols, fluorescence lifetime amounts to 1.35 ns (τ_1) and 3.22 ns (τ_2), which is similar to values found for other proteins.[41,46]

The short-lived and long-lived fluorescence components account for approximately 68% and 32% of the total, respectively. The short-lived component is probably attributable to Trp 193, which resides on the molecule surface, and to Trp 84, located near the surface. In contrast, the long-lived component is likely to be emitted by Trp 310, which is situated deep inside the protein molecule. In the presence of 5 μM RSV, the fluorescence lifetime of GAPDH does not differ from that of GAPDH without the polyphenol. In the presence of 25 μM RSV, fluorescence lifetimes slightly increase. This suggests that at low RSV concentrations, GAPDH fluorescence quenching is a static process, while at higher concentrations also dynamic quenching may be involved. This is consistent with the ζ potential results, which show that the GAPDH−RSV is formed up to RSV concentrations of approximately 6 μM (at higher RSV concentrations the ζ potential remains unchanged). In the case of THMS, which interacts with GAPDH much more weakly, fluorescence lifetimes do not change statistically significantly, which indicates that quenching is static. The considerably smaller changes in the ζ potential corroborate this finding.

ζ Potential. Changes in the ζ potential provide yet another proof for interactions between the studied polyphenols and GAPDH. The ζ potential of GAPDH decreased with increasing molar ratio of the polyphenols to the protein (Figure 6). At a polyphenol-to-protein ratio higher than 6, the potential remained constant. RSV caused a larger decrease in the potential of GAPDH: from −4.7 mV in the absence of the ligand to −9 mV in its presence. The potential of GAPDH declined less dramatically (down to −6.8 mV) in the presence of THMS. The observed changes indicate that the studied polyphenols affect the charge on the GAPDH surface. Changes in the ζ potential are closely correlated to the fluorescence quenching rates described above. They seem to result from polyphenol binding to sites near Trp 84 and Trp 193 and in the region with the greatest accessibility to ligands, that is, in the area where the subunits come into contact.

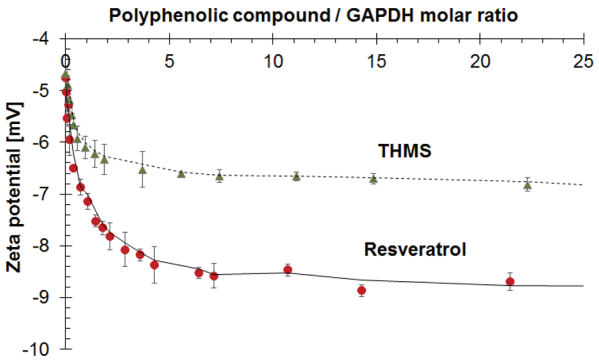

Figure 6. ζ potential of GAPDH in the presence of THMS and resveratrol.

Antioxidant Properties of the Studied Polyphenols. In general, polyphenols are very good antioxidants. Their reaction rate constants with the hydroxyl radical are high, ranging from 10^8 to 10^9 M^{-1} s^{-1}, while the reaction rate constants with the superoxide anion radical ($O_2^{\cdot -}$) amount to 10^6–10^7 M^{-1} s^{-1}.[47−50] However, under some circumstances, polyphenols may also exhibit pro-oxidant properties.[37,38] In our previous paper, we reported that in the presence of the superoxide anion radical RSV increased GAPDH inactivation in a concentration-dependent manner.[37] Therefore, in the current work we studied the effect of THMS on the activity of GAPDH in the presence of the superoxide anion radical to compare it with RSV. The activity of GAPDH exposed to $O_2^{\cdot -}$ was determined in the absence and presence of the polyphenols. The superoxide anion radical was generated chemically in a reaction of xanthine with xanthine oxidase in the presence of catalase. Relative GAPDH activity was determined with respect to the enzyme incubated with the polyphenols in the absence of the superoxide anion radical.

In the absence of the polyphenols, dehydrogenase inactivation increased with increasing amounts of the superoxide anion radical generated (Figure 7). The activity of the enzyme fell by 50% at 64 μM superoxide anion radical. Under the experimental conditions, THMS did not modify the inactivation of GAPDH induced by the superoxide anion radical. In contrast, RSV magnified GAPDH inactivation, and the activity of the enzyme decreased by 50% at approximately 51 μM superoxide anion radical generated. In a parallel experiment, we found that the removal of the superoxide anion

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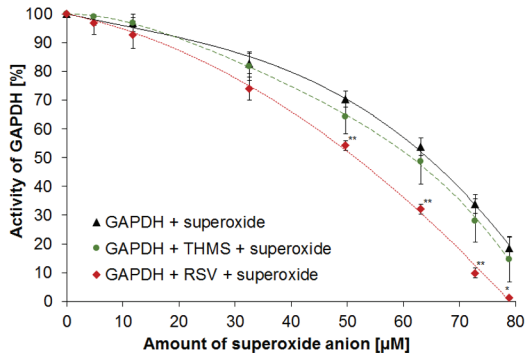

Figure 7. GAPDH inactivation induced by the superoxide anion radical in the absence and presence of THMS or RSV, respectively. Polyphenol concentration was 50 μM. Enzymatic activity was expressed in relative values (%) with respect to the controls: GAPDH activity was the control (100%) for the GAPDH + superoxide system (triangle); GAPDH activity in the presence of the polyphenols was the control (100%) for the GAPDH + polyphenol + superoxide system (circle or rhombus). Data are the mean ± SD of three to five independent measurements. The asterisks indicate a significant difference between activity of GAPDH + superoxide and the activity of GAPDH + polyphenol + superoxide: (*) $p < 0.01$, (**) $p < 0.001$ (t test).

radical by superoxide dismutase, both in the presence and absence of the polyphenols, protected the enzyme from inactivation (data not shown in the figure).

Given the reaction rate constant of $O_2^{\cdot -}$ with RSV (2×10^7 M^{-1} s^{-1}) and a similar rate constant of $O_2^{\cdot -}$ with GAPDH, at the employed concentrations of the dehydrogenase (1.4 μM) and the polyphenols (50 μM), approximately 90−95% of $O_2^{\cdot -}$ reacts with the polyphenols, giving rise to secondary radicals of the latter. The presented study indicates increased inactivation of GAPDH exposed to the superoxide anion radical in the presence of resveratrol. In the process of scavenging superoxide anion radicals, RSV is transformed into a compound with an alkoxy radical at position 4′ of the phenol ring. Stojanović et al. showed that the hydroxyl group at position 4′ (the para-hydroxyl group) scavenges peroxyl radicals more efficiently than the hydroxyl groups at positions 3′ and 5′ (meta-hydroxyl groups).[36] In a similar vein, Rossi et al. observed that $O_2^{\cdot -}$ reacts with the OH group at position 4′, while the likelihood of this radical reacting with other hydroxyl groups in the RSV molecule is much lower. According to Caruso et al., the hydroxyl group at position 4′ is more acidic than the other two OH groups, so the mechanism of free radical scavenging by RSV and its derivatives mostly implies a loss of the hydrogen atom from position 4′.[26]

In contrast to RSV, THMS did not magnify GAPDH inactivation induced by the superoxide ion radical. The position and number of OH groups are the main determinants of the antioxidant properties of stilbenes. THMS differs from resveratrol in that in its B ring it has not only two OH groups but also a methoxy group at position 4′. As Stivala et al. reported, partial methylation of hydroxyl groups decreases the antioxidant properties of resveratrol, while complete methylation may result in a total loss of antioxidant activity.[35] Thus, it may be expected that THMS exhibits lower reactivity with the superoxide anion radical than with RSV. As the reaction rate constant of $O_2^{\cdot -}$ with THMS is not known, it is difficult to estimate what proportion of radicals of this compound react with GAPDH. Therefore, it is impossible to determine whether GAPDH is inactivated by phenoxyl radicals of THMS or directly by the superoxide anion radical.

The obtained results show that RSV exhibits pro-oxidant properties, that is, the RSV radical inactivates GAPDH to a greater extent than the superoxide anion radical. On the other hand, even if the THMS radical is created as a result of reaction with the superoxide anion radical, then its efficiency of inactivating the studied enzyme is the same as that of the superoxide anion radical itself.

CONCLUSIONS

Both the structural and functional changes induced by the studied polyphenols are attributable to their structure. Because of the methoxy group at position 4′ in the phenyl ring of stilbene, THMS binds to GAPDH less readily than resveratrol. However, THMS binds more strongly within the active site, forming more hydrogen bonds, which decreases the activity of GAPDH. In resveratrol, of critical importance to its pro-oxidant properties is the OH group at position 4′. While resveratrol magnifies the inactivation of GAPDH induced by the superoxide anion radical, THMS has no effect on that inactivation.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RSV or resveratrol, 3,5,4′-trihydroxystilbene; THMS, *trans*-3,3′,5,5′-tetrahydroxy-4′-methoxystilbene; D-S, value of the dock-score function; $O_2^{\cdot -}$, superoxide anion radical

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na zaprojektowaniu przeprowadzonych badań, naświetlaniu roztworów badanych białek promieniami X oraz wykonaniu dozymetrii metodą Frickego. Wykonałam pomiary dichroizmu kołowego roztworów GAPDH i LDH poddanych działaniu promieniowania X w obecności lub pod nieobecność badanych przeciwutleniaczy. Napisałam pierwotną wersję manuskryptu oraz byłam odpowiedzialna za korespondencję z redakcją czasopisma i recenzentami.

Mój wkład w powstanie pracy oceniam na 40%.

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na wykonaniu pomiarów aktywności enzymów (GAPDH, LDH) poddanych działaniu promieniowania X bez przeciwutleniaczy (w warunkach nieredukujących jak i redukujących) oraz w obecności melatoniny i resweratrolu. Oznaczyłam zawartość wolnych reszt tiolowych metodą Ellmana w GAPDH i LDH poddanych działaniu promieniowania X w obecności przeciwutleniaczy lub pod ich nieobecność. Opracowałam uzyskane wyniki. Ponadto przygotowałam roztwory białek i przeciwutleniaczy do rozdziałów chromatograficznych, uczestniczyłam w przeprowadzanych rozdziałach jak i w opracowaniu wyników otrzymanych metodą HPLC. Brałam udział w korekcie artykułu przed wysłaniem do wydawnictwa oraz w tłumaczeniu artykułu na język angielski.

Mój wkład w powstanie pracy oceniam na 40%.

Joanna Strumiłło

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na określeniu celu i rodzaju badań, naświetlaniu roztworów badanych enzymów promieniami X oraz wykonaniu dozymetrii metodą Frickego. Przeprowadziłem rozdziały chromatograficzne naświetlanych enzymów w obecności lub pod nieobecność resweratrolu i melatoniny oraz opracowałem otrzymane wyniki. Uczestniczyłem w przygotowaniu pierwotnej wersji artykułu.

Mój wkład w powstanie pracy oceniam na 10%.

Mieczysław Puchała

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na określeniu metodą modelowania molekularnego potencjalnych miejsc wiązania melatoniny i resweratrolu do cząsteczek GAPDH i LDH. Określiłem ilość miejsc wiązania i przygotowałem graficzne podsumowanie wyników. Opracowałem statystycznie wyniki badań przeprowadzonych w niniejszej publikacji.

Mój wkład w powstanie pracy oceniam na 5%.

Eligiusz Serafin

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Puchala, M., Serafin, E., Bartosz, G. (2019). Comparison of protective properties of resveratrol and melatonin in the radiation inactivation and destruction of glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase. *International Journal of Radiation Biology*, *95*(11), 1472-1483.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na ocenie manuskryptu przed jego wysłaniem oraz przygotowaniu odpowiedzi dla recenzentów.

Mój wkład w powstanie pracy oceniam na 5%.

G Bartosz

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na optymalizacji użytej w pracy metody otrzymywania gazowego tlenku azotu (NO). Izolowałam, a następnie oczyszczałam metodą chromatograficzną hemoglobinę użytą do określenia stężenia NO w przeprowadzonych eksperymentach. Przygotowałam roztwory badanych białek poddanych działaniu tlenku azotu do dalszych oznaczeń. Zaprojektowałam część wykonanych badań. Określiłam aktywność oraz zawartość wolnych reszt tiolowych w enzymach poddanych działaniu NO w obecności lub pod nieobecność przeciwutleniaczy. Przeprowadziłam badanie odwracalności modyfikacji GAPDH z udziałem NO oraz oszacowałam potencjalne miejsca S-nitrozylacji GAPDH i LDH przy użyciu programu GPS-SNO 1.0. Opracowałam uzyskane wyniki oraz napisałam pierwotną wersję publikacji. Byłam odpowiedzialna za korespondencję z redakcją czasopisma oraz przygotowanie odpowiedzi dla recenzentów.

Mój wkład w powstanie pracy oceniam na 55%.

Joanna Strumiłło

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na wykonaniu oznaczeń zmian strukturalnych GAPDH i LDH zachodzących pod wpływem tlenku azotu w obecności i pod nieobecność melatoniny i resweratrolu z użyciem znacznika fluorescencyjnego bis-ANS. Opracowałam otrzymane wyniki oraz napisałam część dotyczącą metodyki wykonanych oznaczeń. Dokonałam korekty manuskryptu przed jego wysłaniem do druku.

Mój wkład w powstanie pracy oceniam na: 5%.

Katarzyna Nowak

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na oznaczeniu stężenia produktów utlenienia tlenku azotu w obecności badanych białek metodą spektrofotometryczną. Opracowałam otrzymane wyniki oraz napisałam część dotyczącą metodyki przeprowadzonych przeze mnie badań. Dokonałam korekty artykułu przed jego wysłaniem do czasopisma.

Mój wkład w powstanie pracy oceniam na 5%.

Anita Krokosz

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na zaprojektowaniu większości przeprowadzonych badań oraz recenzji pracy.

Mój wkład w powstanie pracy oceniam na 20%.

Aleksandra Rodacka

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na zaprojektowaniu większości przeprowadzonych badań, w tym zmodyfikowanej metody otrzymywania gazowego tlenku azotu oraz metody oznaczania jego stężenia w badanych układach. Byłem także odpowiedzialny za opracowanie statystyczne otrzymanych wyników. Dokonałem korekty artykułu przed jego wysłaniem do czasopisma.

Mój wkład w powstanie pracy oceniam na 10%.

Mieczysław Puchała

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Strumillo, J., Nowak, K. E., Krokosz, A., Rodacka, A., Puchala, M., Bartosz, G. (2018). The role of resveratrol and melatonin in the nitric oxide and its oxidation products mediated functional and structural modifications of two glycolytic enzymes: GAPDH and LDH. *Biochimica et Biophysica Acta - General Subjects*, *1862*(4), 877-885.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na recenzji pracy i korekcie językowej.

Mój wkład w powstanie pracy oceniam na 5%.

GBartosz

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Serafin, E., Puchala, M. (2015). Analysis of Potential Binding Sites of 3,5,4'-Trihydroxystilbene (Resveratrol) and trans-3,3',5,5'-Tetrahydroxy-4'-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols. *Journal of Physical Chemistry B*, *119*(30), 9592-9600.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na zaproponowaniu ogólnego celu przeprowadzonych badań. Wykonałam doświadczenia służące do zbadania oddziaływań GAPDH z badanymi związkami poprzez pomiar zmian potencjału zeta GAPDH. Opracowałam otrzymane wyniki. Przygotowałam pierwotną wersję manuskryptu oraz prowadziłam korespondencję z redakcją czasopisma i recenzentami.

Mój wkład w powstanie pracy oceniam na 50%.

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ul. Pomorska 141/143, 90-236 Łódź

Łódź, 28.09.2020 r.

Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Serafin, E., Puchala, M. (2015). Analysis of Potential Binding Sites of 3,5,4'-Trihydroxystilbene (Resveratrol) and trans-3,3',5,5'-Tetrahydroxy-4'-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols. *Journal of Physical Chemistry B*, *119*(30), 9592-9600.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na wykonaniu pomiarów aktywności GAPDH w obecności badanych związków polifenolowych, a także w obecności związków i anionorodnika ponadtlenkowego oraz opracowaniu otrzymanych wyników. Zaproponowałam sposób wykonania i przeprowadziłam eksperymenty związane z określeniem interakcji GAPDH z badanymi związkami poprzez pomiar gaszenia fluorescencji białka. Wstępnie opracowałam otrzymane wyniki służące do wyznaczenia stałych Sterna-Volmera. Brałam udział w pisaniu manuskryptu publikacji.

Mój wkład w powstanie pracy oceniam na 40%.

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Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Serafin, E., Puchala, M. (2015). Analysis of Potential Binding Sites of 3,5,4'-Trihydroxystilbene (Resveratrol) and trans-3,3',5,5'-Tetrahydroxy-4'-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols. *Journal of Physical Chemistry B*, *119*(30), 9592-9600.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na określeniu metodą modelowania molekularnego potencjalnych miejsc wiązania badanych związków polifenolowych do cząsteczki GAPDH. Określiłem położenie najbardziej prawdopodobnych miejsc wiązania badanych ligandów, ilość miejsc wiązania, rodzaj oddziaływań z sąsiadującymi resztami aminokwasowymi. Określiłem położenie reszt tryptofanu w sąsiedztwie miejsc wiązania polifenoli. Opracowałem graficznie otrzymane wyniki. Zaproponowałem i wykonałem korektę wyników gaszenia fluorescencji GAPDH w celu wyznaczenia stałych Sterna-Volmera.

Mój wkład w powstanie pracy oceniam na 5%.

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Łódź, 28.09.2020 r.

Oświadczenie

Niniejszym oświadczam, iż jestem współautorem publikacji naukowej: Rodacka, A., Strumillo, J., Serafin, E., Puchala, M. (2015). Analysis of Potential Binding Sites of 3,5,4'-Trihydroxystilbene (Resveratrol) and trans-3,3',5,5'-Tetrahydroxy-4'-methoxystilbene (THMS) to the GAPDH Molecule Using a Computational Ligand-Docking Method: Structural and Functional Changes in GAPDH Induced by the Examined Polyphenols. *Journal of Physical Chemistry B, 119*(30), 9592-9600.

Wyrażam zgodę na włączenie wyżej wymienionej pracy do cyklu publikacji stanowiących rozprawę doktorską mgr Joanny Strumiłło.

Mój wkład w powstanie publikacji polegał na określeniu celu i rodzaju przeprowadzonych badań. Przeprowadziłem eksperymenty określające czas życia fluorescencji reszt tryptofanu w cząsteczce GAPDH w obecności i pod nieobecność badanych związków polifenolowych. Uczestniczyłem w przygotowaniu pierwotnej wersji artykułu.

Mój wkład w powstanie pracy oceniam na 5%.

Mieczysław Puchała