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Wpływ bisfosfonianów stosowanych w terapii osteoporozy na metabolizm tlenowy komórek śródbłonka

The effects of bisphosphonates used in osteoporosis therapy on aerobic metabolism of endothelial cells

Praca doktorska wykonana pod kierunkiem
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Spis treści

1. Streszczenie

Osteoporoza jest najczęstszą chorobą metabolizmu kości, głównie wśród kobiet w wieku pomenopauzalnym. Najczęściej stosowanymi lekami zmniejszającymi resorpcję kości są bisfosfoniany zawierające azot (N-BPs). Mechanizm działania N-BPs opiera się na blokowaniu enzymu syntazy farnesylopirofosforanu (FPPS) w szlaku mewalonowym. Szlak mewalonowy odgrywa ważną rolę w wielu procesach komórkowych takich jak prenylacja białek i modyfikacje potranslacyjne a także synteza cząsteczek zawierających jednostki izoprenoidowe, w tym hemów *a* (grup prostetycznych kompleksu IV) czy koenzymu Q (CoQ). CoQ jest kluczowym przenośnikiem elektronów w mitochondrialnym łańcuchu oddechowym oraz ważnym przeciwutleniaczem chroniącym przed uszkodzeniami peroksydacyjnymi, obecnym we wszystkich błonach komórki, głównie mitochondrialnych. Komórki śródbłonka wyścielają naczynia krwionośne, co sprawia, że mają ciągły kontakt z substancjami transportowanymi przez krew, w tym lekami.

Praca doktorska koncentruje się na wieloaspektowym wpływie N-BPs na metabolizm tlenowy komórek śródbłonka naczyń krwionośnych. Rozprawa złożona jest z dwóch prac doświadczalnych i jednej pracy przeglądowej, które prezentują analizę mechanizmów za pomocą których N-BPs, takie jak alendronian i zoledronian, wpływają na metabolizm energetyczny, stres oksydacyjny i funkcjonowanie mitochondriów w komórkach.

Materiałem badawczym użytym w pracach doświadczalnych były ludzkie komórki śródbłonka EA.hy926, traktowane przez sześć dni 5 μM alendronianem lub 1 μM zoledronianem, oraz izolowane mitochondria z tych komórek. Analiza zmian metabolizmu energetycznego wywołanych przez N-BPs została przeprowadzona w dwóch etapach tj. na poziomie komórkowym (Publikacja 1) oraz mitochondrialnym (Publikacja 2).

Doświadczenia przeprowadzone na całych komórkach śródbłonka (Publikacja 1) pokazały, że N-BPs wywołują spadek żywotności komórek, nasilony stres oksydacyjny i stan zapalny oraz obniżenie aktywności szlaku sygnałowego ERK1/2 zależnego od prenylacji. Stwierdzono także istotne zmiany w metabolizmie energetycznym komórek polegające na zwiększeniu oddychania beztlenowego, obniżeniu mitochondrialnego utleniania głównych substratów oddechowych (oprócz kwasów tłuszczowych) i spadku poziomu ATP. Po raz pierwszy pokazano, że zahamowanie szlaku mewalonowego przez N-BPs wywołuje w komórkach śródbłonka znaczny spadek poziomu całkowitego CoQ oraz utratę jego zredukowanej puli. Zmianom tym towarzyszy wzrost produkcji reaktywnych form tlenu (RFT).

Badania wskazują, że N-BPs zmieniają także dynamikę mitochondriów poprzez redukcję ich fragmentacji. A zatem, N-BPs modulują metabolizm energetyczny komórek śródbłonka prowadząc do zmian w statusie energetycznym komórek, homeostazie redoks CoQ, aktywności metabolizmu tlenowego i kontroli jakości mitochondriów.

Kolejne badania prowadzone na poziomie mitochondriów izolowanych z komórek śródbłonka poddanych sześciodniowej hodowli z N-BPs (Publikacja 2) koncentrowały się na adaptacji bioenergetyki mitochondriów do dużego ponad 40% spadku poziomu mitochondrialnego CoQ (mtCoQ) wywołanego zahamowaniem szlaku mewalonowego. W mitochondriach komórek traktowanych N-BPs w porównaniu do mitochondriów kontrolnych, obniżeniu całkowitej puli i utracie zredukowanej puli mtCoQ towarzyszy większa produkcja RFT i aktywacja systemów antyoksydacyjnych. Niedobór mtCoQ prowadzi do obniżonego utleniania substratów oddechowych (oprócz kwasów tłuszczowych), obniżonej aktywności kompleksów II i III oraz syntazy ATP, spadku potencjału błonowego mitochondriów, efektywności syntezy ATP oraz reorganizacji superkompleksów łańcucha oddechowego. Większa produkcja RFT związana jest z większym poziomem redukcji mtCoQ. Po raz pierwszy zaobserwowano, że N-BPs mogą prowadzić do obniżenia poziomu hemów *a* w mitochondriach. A zatem wyniki te wskazują, że wywołane przez N-BPs zaburzenie homeostazy redoks mtCoQ może istotnie wpływać na aktywność bioenergetyczną mitochondriów komórek śródbłonka.

Praca przeglądowa przedstawiona w ramach rozprawy (Publikacja 3) zbiera wyniki dotychczasowych badań, wskazujące na szerokie spektrum działania N-BPs poza tkanką kostną – w tym ich wpływ na organizację cytoszkieletu, apoptozę zależną od mitochondriów, gospodarkę wapniową oraz procesy związane z kontrolą jakości mitochondriów. N-BPs, jako inhibitory szlaku mewalonowego, powodują szereg skutków ubocznych zarówno na poziomie całej komórki jak i dotyczących funkcjonowania mitochondriów. Dalsze badania nad złożonym powiązaniem pomiędzy szlakiem mewalonowym, funkcjonowaniem mitochondriów a ogólną homeostazą komórkową mogą być istotne dla optymalizacji klinicznego zastosowania N-BPs, być może uwzględniającego suplementację CoQ10, i ograniczenia efektów ubocznych w tkankach niebędących celem terapii.

Wnioski z pracy doktorskiej podkreślają istotne, potencjalnie niepożądane skutki działania N-BPs na komórki niezwiązane z kośćcem, na przykładzie komórek śródbłonka. Przedstawione badania sugerują, że efekty te mogą przyczyniać się do powikłań sercowo-naczyniowych u pacjentów leczonych N-BPs, a mechanizmy mitochondrialne mogą stanowić istotny punkt docelowy dalszych badań oraz potencjalnych strategii ochronnych.

2. Summary

Osteoporosis is the most common disease of bone metabolism, mainly among postmenopausal women. The most commonly used drugs to reduce bone resorption are nitrogen-containing bisphosphonates (N-BPs). The mechanism of action of N-BPs is based on blocking the enzyme farnesylpyrophosphate synthase (FPPS) in the mevalonate pathway. The mevalonate pathway plays an important role in many cellular processes such as protein prenylation and post-translational modifications as well as the synthesis of molecules containing isoprenoid units, including *a*-hemes (prosthetic groups of complex IV) or coenzyme Q (CoQ). CoQ is a key electron carrier in the mitochondrial respiratory chain and an important antioxidant present in all cell membranes, mainly mitochondrial, that protects against peroxidative damage. Endothelial cells line blood vessels, which means they are in constant contact with substances transported by the blood, including drugs.

The doctoral thesis focuses on the multifaceted influence of N-BPs on the oxidative metabolism of vascular endothelial cells. The dissertation consists of two experimental papers and one review paper, which present an analysis of the mechanisms by which N-BPs, such as alendronate and zoledronate, affect energy metabolism, oxidative stress and mitochondrial function in cells.

The research material used in the experiments were human endothelial cells EA.hy926, treated for six days with 5 μM alendronate or 1 μM zoledronate, and isolated mitochondria from these cells. The analysis of changes in energy metabolism induced by N-BPs was carried out in two stages, i.e., at the cellular level (Publication 1) and the mitochondrial level (Publication 2).

Experiments conducted on whole endothelial cells (Publication 1) showed that N-BPs cause a decrease in cell viability, increased oxidative stress and inflammation, and a decrease in the activity of the prenylation-dependent ERK1/2 signaling pathway. Significant changes in cell energy metabolism were also observed, consisting in increased anaerobic respiration, decreased mitochondrial oxidation of the main respiratory substrates (except fatty acids) and a decrease in ATP level. For the first time, it has been shown that inhibition of the mevalonate pathway by N-BPs induces a significant decrease in the level of total CoQ and a loss of its reduced pool in endothelial cells. These changes are accompanied by an increase in the production of reactive oxygen species (ROS). Studies indicate that N-BPs also change the dynamics of mitochondria by reducing their fission. Thus, N-BPs modulate the energy

metabolism of endothelial cells leading to changes in the energy status of cells, CoQ redox homeostasis, the activity of oxygen metabolism and mitochondrial quality control.

Further studies conducted at the level of mitochondria isolated from endothelial cells cultured for six days with N-BP (Publication 2) focused on the adaptation of mitochondrial bioenergetics to the large decrease of more than 40% in mitochondrial CoQ (mtCoQ) levels caused by the inhibition of the mevalonate pathway. In mitochondria of cells treated with N-BPs, compared with control mitochondria, the decrease in the total pool and the loss of the reduced pool of mtCoQ are accompanied by greater production of ROS and activation of antioxidant systems. mtCoQ deficiency leads to decreased oxidation of respiratory substrates (except fatty acids), decreased activity of complexes II and III and ATP synthase, decreased mitochondrial membrane potential, efficiency of ATP synthesis and reorganization of respiratory chain supercomplexes. Increased ROS production is associated with higher reduction level of mtCoQ. It was observed for the first time that N-BPs can lead to decreased level of heme *a* in mitochondria. Therefore, these results indicate that N-BP-induced disruption of mtCoQ redox homeostasis can significantly affect bioenergetic activity of endothelial mitochondria.

The review presented in the dissertation (Publication 3) summarizes the results of previous studies indicating a wide spectrum of N-BP action outside bone tissue – including their effects on cytoskeletal organization, mitochondrial-dependent apoptosis, calcium metabolism and processes related to mitochondrial quality control. N-BPs, as inhibitors of the mevalonate pathway, cause a number of side effects both at the cellular level and mitochondrial function. Further studies on the complex relationship between the mevalonate pathway, mitochondrial function and overall cellular homeostasis may be important to optimize the clinical use of N-BPs, possibly including CoQ10 supplementation, and to limit side effects in off-target tissues.

The conclusions of the doctoral thesis highlight the significant, potentially adverse effects of N-BP on non-skeletal cells, exemplified by endothelial cells. The studies presented suggest that these effects may contribute to cardiovascular complications in patients treated with N-BP, and mitochondrial mechanisms may be an important target for further research and potential protective strategies.

3. Wykaz publikacji wchodzących w skład rozprawy doktorskiej

Publikacja 1:

Budzinska A, Galganski L, Jarmuszkiewicz W (2023) The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells, *Scientific Reports*, 13(1), (IF_{5y} 4,3), doi: 10.1038/s41598-023-43377-3

Publikacja 2:

Budzinska A, Galganski L, Wojcicki K, Jarmuszkiewicz W (2025) Adaptation of mitochondrial bioenergetic activity associated with coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates, alendronate and zoledronate, *Scientific Reports*, 15, (IF_{5y} 4,3), doi: 10.1038/s41598-025-02710-8

Publikacja 3:

Budzinska A, Jarmuszkiewicz W (2025) Consequences of inhibition of mevalonate pathway by bisphosphonates at cellular and mitochondrial levels, manuskrypt wysłany (16.06.2025) do czasopisma *Pharmaceuticals*, w recenzji, zdeponowany w preprints.org, doi: 10.20944/preprints202506.2071.v1

4. Wprowadzenie

Osteoporoza jest najczęstszą chorobą metabolizmu kości, głównie wśród kobiet w wieku pomenopauzalnym. Wraz ze zwiększeniem liczby starszych osób w społeczeństwie mówi się o powstawaniu zjawiska globalnej epidemii osteoporozy. Już 10 lat temu oceniano, iż osteoporozą jest dotkniętych 200 milionów osób na świecie, w tym co trzecia kobieta powyżej 50 roku życia [1]. Proces przebudowywania kości opiera się głównie na trzech typach komórek: osteoblastach nadbudowujących kości, osteoklastach resorbujących kości oraz osteocytach. W wyniku działania czynników genetycznych i środowiska równowaga między nadbudowywaniem i resorpcją kości może być zaburzona.

W leczeniu osteoporozy najpopularniejszym podejściem jest zmniejszenie tempa resorpcji kości. Najczęściej stosowanymi lekami antyresorpcyjnymi są bisfosfoniany. Leki te dzielą się na dwie grupy: niezawierające azot oraz zawierające azot (N-BPs). Lecznicze działanie N-BPs opiera się na blokowaniu enzymu syntazy farnezylopirofosforanu (FPPS) w szlaku mewalonowym w komórkach osteoklastów. Prenylacja białek będąca efektem szlaku mewalonowego jest niezbędna do osiągnięcia aktywności resorpcyjnej osteoklastów. Zahamowanie tego szlaku powoduje zwiększoną apoptozę osteoklastów, zmniejszając tym samym tempo resorpcji kości [2].

Szlak mewalonowy odgrywa ważną rolę w wielu procesach komórkowych takich jak prenylacja białek i modyfikacje potranslacyjne oraz synteza cząsteczek zawierających jednostki izoprenoidowe, w tym cholesterolu, hemów *a* (grup prostetycznych kompleksu IV) czy koenzymu Q (CoQ), obecnego we wszystkich błonach komórkowych, głównie w wewnętrznej błonie mitochondrialnej. CoQ to rozpuszczalny w tłuszczach, wytwarzany endogennie przeciwutleniacz, chroniący przed stresem oksydacyjnym i peroksydacją lipidów, białek czy DNA [3]. Neutralizując wolne rodniki, takie jak anionorodnik ponadtlenkowy, zredukowana forma CoQ ($CoQH_2$) zapobiega rozprzestrzenianiu się stresu oksydacyjnego [4]. Ponadto, $CoQH_2$ wspomaga regenerację puli innych przeciwutleniaczy, takich jak witamina E i C, poprzez redukcję ich utlenionych form. Duży potencjał antyoksydacyjny CoQ wynika z jego obfitego rozmieszczenia w różnych błonach komórkowych oraz efektywnych procesów redukcji odnawiających jego zredukowaną pulę [3].

Główną funkcją CoQ jest transport elektronów w mitochondrialnym łańcuchu oddechowym i tym samym zasilanie produkcji ATP. Mitochondrialny CoQ (mtCoQ) przenosi elektrony między dehydrogenazami (kompleksem I i II) i utleniającym go kompleksem III,

stanowiąc istotny etap w procesie fosforylacji oksydacyjnej. Jednakże, mtCoQ może także przyczyniać się do powstawania reaktywnych form tlenu w mitochondriach (RFT). Przy niepełnej redukcji mtCoQ, dochodzi do powstania rodnika semichinonowego, który wchodząc w interakcję z tlenem tworzy anionorodnik ponadtlenkowy, będący prekursorem innych RFT. Nadmiar RFT prowadzi do stresu oksydacyjnego, który może przyczyniać się do starzenia komórek czy powstawania różnych chorób [4].

Biodostępność N-BPs podczas podania doustnego jest bardzo niska (~1%). 99% przyjętego leku jest usuwane z organizmu. W związku z tym, w cięższych przypadkach chorób kostnych, zaleca się dożylne podawanie tych terapeutyków. N-BPs silnie wiążą się do hydroksyapatytu, obecnego w miejscach o zwiększonej resorpcji kości. Podczas procesu resorpcji N-BPs są uwalniane do krwiobiegu w celu wydalenia z moczem. W zależności od typu N-BP i siły jego penetracji w głąb kości, jego uwalnianie może trwać nawet do 10 lat po zakończonej terapii [5]. Dlatego też, komórkami mającymi długotrwały kontakt z tymi lekami są komórki śródbłonka czy nerek.

Śródbłonek wyścieła wszystkie naczynia krwionośne, od serca do naczyń włosowatych. Kontroluje przepływ substancji do i z krwiobiegu. Wszystkie tkanki są zależne od dostępu do krwi, czyniąc je zależnymi od komórek śródbłonka. Dysfunkcja tych komórek wpływa na wiele tkanek i narządów oraz jest podłożem wielu chorób cywilizacyjnych [6]. Obecnie coraz więcej uwagi poświęca się roli mitochondriów w procesach patofizjologicznych obejmujących dysfunkcję śródbłonka. Komórki śródbłonka, opierające swój metabolizm głównie na glikolizie, posiadają mniejszą niż komórki innych tkanek liczbę mitochondriów, działających przede wszystkim jako organelle sygnałowe. Dysfunkcja komórek śródbłonka jest powiązana z reumatoidalnym zapaleniem stawów, cukrzycą, hipercholesterolemią czy chorobą wieńcową.

Mitochondria są organellami odpowiedzialnymi za uzyskiwanie energii w procesie fosforylacji oksydacyjnej, zlokalizowanym w wewnętrznej błonie mitochondrialnej. Proces jest niezbędny do produkcji ATP, który zapewnia energię niezbędną do prawidłowego funkcjonowania komórki. Oprócz tego mitochondria biorą udział w apoptozie, sygnalizacji komórkowej oraz uczestniczą w procesach buforowania wapnia [7]. Zaburzenie delikatnej homeostazy w mitochondriach może prowadzić do zmniejszenia żywotności komórek poprzez uwolnienie cytochromu *c* i następnie aktywację mitochondrialnej ścieżki apoptozy [8]. Mitochondria to dynamiczne organelle podlegające ciągłym procesom fragmentacji, fuzji, biogenezie czy mitofagii co wpływa na ich funkcjonowanie. Badania dysfunkcji

mitochondriów różnych komórek, wynikającej z działania N-BPs, pozwoliłyby wyjaśnić procesy patofizjologiczne efektów ubocznych stosowania terapii antyresorpcyjnej.

N-BPs, hamując szlak mewalonowy, oprócz pożądanego zmniejszania prenylacji białek kluczowych dla aktywności osteoklastów, mogą obniżać syntezę CoQ. Dotąd odnotowano jedynie obniżony poziom CoQ w surowicy kobiet w wieku pomenopauzalnym, leczonych N-BPs [9]. Brakuje badań opisujących obniżony przez N-BPs poziom CoQ w komórkach, tkankach czy narządach. Podobnie nikt wcześniej nie badał wpływu N-BPs na poziom mtCoQ i wynikające z niego konsekwencje metaboliczne i energetyczne. Dotąd nie badano dysfunkcji mitochondriów wywołanej przez N-BPs, ze szczególnym uwzględnieniem funkcjonowania łańcucha oddechowego oraz mechanizmów kontroli jakości mitochondriów. Niezbadane jest także powiązanie hamowanie szlaku mewalonowego przez N-BPs z funkcjonowaniem mitochondriów i ogólną homeostazą energetyczną komórkową komórek innych kostne.

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5. Cele pracy

Celem pracy doktorskiej było zbadanie wpływu dwóch popularnych N-BPs, zoledronianu (Zol) i alendronianu (Ale) na metabolizm tlenowy komórek śródbłonka. Materiałem użytym w badaniach były komórki ludzkiego śródbłonka EA.hy926 oraz izolowane z nich mitochondria. Komórki hodowano przez 6 dni w obecności 5 µM Ale lub 1 µM Zol.

W ramach badań zrealizowano następujące zadania:

1. Opis zmian w metabolizmie tlenowym komórek śródbłonka hodowanych w obecności N-BPs w odniesieniu do zawartości CoQ, produkcji RFT oraz dynamiki mitochondriów – badania na poziomie komórkowym (Publikacja 1)
2. Opis zmian w aktywności bioenergetycznej mitochondriów izolowanych z komórek śródbłonka hodowanych w obecności N-BPs w odniesieniu do zawartości/stopnia redukcji mtCoQ, produkcji mtRFT oraz molekularnej organizacji łańcucha oddechowego – badania na poziomie izolowanych mitochondriów (Publikacja 2)
3. Zebranie aktualnych danych literaturowych na temat wpływu hamowania szlaku mewalonowego przez N-BPs na poziomie komórkowym i mitochondrialnym (Publikacja 3)

Badania na poziomie komórkowym i mitochondrialnym (Zadanie 1 i 2) pozwoliły na pełniejszy obraz zmian w funkcjonowaniu mitochondriów w komórkach śródbłonka wywołanych przez N-BPs. Badania te pokazują, w jaki sposób komórki śródbłonka, które mają niewielką ilość mitochondriów oraz są pierwszymi komórkami narażonymi in vivo na dożylnie podawane N-BPs (na przykład podczas leczenia osteoporozy), przystosowują się do chronicznego stresu oksydacyjnego wywołanego przez te związki poprzez hamowanie szlaku mewalonowego.

6. Wyniki

Publikacja 1: Budzinska A, Galganski L, Jarmuszkiewicz W, (2023), The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells, *Scientific Reports*, 13(1), (IF_{5y} 4,3), doi: 10.1038/s41598-023-43377-3

W pracy tej opisano zmiany w metabolizmie tlenowym komórek śródbłonka EA.hy926 hodowanych w obecności Zol lub Ale przez 6 dni. Stosowane w tych badaniach N-BPs zostały wybrane ze względu na popularność w terapii antyresorpcyjnej (Ale) oraz ze względu na szczególnie silne działanie (Zol). Mikromolarne stężenia N-BPs użyte w wykonanych badaniach zostały wybrane na podstawie danych literaturowych podających stężenia tych substancji w osoczu pacjentów przyjmujących bisfosfoniany w ramach terapii osteoporozy.

W testach żywotności użyte zostały stężenia Ale w przedziale 1-10 µM i 0,5-5 µM dla Zol. Wyższe stężenia (od 7,5 µM dla Ale i 2,5 µM dla Zol) obniżały żywotność komórek śródbłonka. Zbadano również poziom CoQ (CoQ10) w komórkach śródbłonka hodowanych w obecności N-BPs. Stężenia 1 µM Zol i 5 µM Ale wywoływały ~30% spadek poziomu CoQ. Do dalszych badań wybrano te stężenia, ponieważ dawały istotny spadek poziomu CoQ, jednocześnie nie wykazując działania na żywotność komórek śródbłonka. Do tej pory, opublikowane badania pokazywały jedynie spadek CoQ w osoczu pacjentek w wieku pomenopauzalnym przyjmujących N-BPs. Natomiast w tych badaniach wykazano po raz pierwszy dla komórek, nie tylko komórek śródbłonka, obniżenie poziomu CoQ związane z zastosowaniem N-BPs.

W komórkach hodowanych w obecności N-BPs obniżeniu poziomu całkowitego CoQ (formy zredukowanej plus utlenionej) towarzyszył zanik zredukowanej formy – $CoQH_2$, która w komórkach kontrolnych wynosiła ~8% całej puli CoQ. W wyniku całkowitego zaniku zredukowanej formy, która działa jako antyoksydant, w komórkach traktowanych doszło do zwiększenia produkcji RFT, zarówno mitochondrialnych jak i komórkowych. W komórkach traktowanych N-BPs, odnotowano również 15-20% wzrost poziomu białek antyoksydacyjnych tj. dysmutazy ponadtlenkowej (SOD1) czy reduktazy glutationowej (GR), co wskazuje na niewystarczającą pulę antyoksydantów w komórkach traktowanych N-BPs i konieczność uruchomienia dodatkowych mechanizmów w celu niwelowania powstałego przez N-BPs stresu oksydacyjnego.

Nasze badania pokazały, że 1 μM Zol zwiększył w komórkach śródbłonka poziom markerów zapalnych: cząsteczki adhezji międzykomórkowej 1 (ICAM1) i interleukiny 6 (IL-6), jednak 5 μM Ale nie wywołał żadnych zmian. Co więcej, w obu typach komórek traktowanych N-BPs o ~20% spadł poziom markera hipoksji tj. czynnika indukowanego hipoksją 1α (HIF1α). Ponadto w komórkach tych aż 6-krotnie wzrósł poziom lizyno-specyficznej demetylazy 6A (KDM6A), działającej jak sensor tlenu regulujący transkrypcję genów. Wyniki te wskazują, że w warunkach zwiększonego poziomu tlenu, o czym świadczy obniżony poziom HIF1α, może dochodzić do zależnych od KDM6A zmian w poziomie białek kontrolujących los komórki oraz organizacji chromatyny. W tej sytuacji zwiększony poziom tlenu, prawdopodobnie w wyniku obniżenia zużycia tlenu przez metabolizm tlenowy, może prowadzić do zwiększenia stresu oksydacyjnego i adaptacji metabolizmu tlenowego komórki.

Przeprowadzona analiza enzymów syntazy cytrynianowej (CS) oraz kompleksu IV (COX) wykazała wzrost zarówno aktywności jak i poziomu obu enzymów (~12-17%), świadczący o zwiększonej pojemności oddechowej cyklu Krebsa i łańcucha oddechowego komórek traktowanych N-BPs. Co ciekawe, zmianom tym towarzyszył ~16-20% wzrost poziomu i ~25-35% wzrost aktywności dehydrogenazy mleczanowej (LDH), a także ~27% wzrost poziomu heksokinazy-1 (HK1). Adaptacje te sprzyjające zwiększeniu produkcji ATP, zarówno poprzez metabolizm tlenowy i beztlenowy, okazały się niewystarczające, ponieważ w komórkach poddanych działaniu N-BPs poziom ATP był niższy niż w komórkach kontrolnych.

Zwiększenie aktywności białek CS oraz COX o ~17% oraz ich poziomu w komórkach poddanych działaniu N-BPs sugeruje, iż zawartość mitochondriów w tych komórkach jest większa. Poziomy białek kanału anionoselektywnego zależnego od napięcia 1 (VDAC1) oraz mitochondrialnego nieglikozylowanego białka (MT) były podwyższone o ~10-15%. Nie zaobserwowano żadnych zmian w poziomie białek charakterystycznych dla biogenezy mitochondriów tj. PGC1α (ang. *peroxisome proliferator-activated receptor gamma coactivator 1-alpha*) i NRF2 (ang. *nuclear factor erythroid 2-related factor 2*). Jednocześnie, poziomy markerów fragmentacji mitochondriów znacząco wzrosły. Poziom czynnika fragmentacji mitochondriów (MFF, ang. *mitochondrial cleveage factor*) wzrósł o ~25%, a poziom fosforylowanego białka zależnego od dynaminy (fosfo-DRP1) wzrósł o ~12%, podczas gdy poziom markera fuzji tj. OPA1 (ang. *arthrosis protein-1*) pozostał na tym samym poziomie co w komórkach kontrolnych. Komórki poddane działaniu N-BPs posiadały również obniżony poziom fosforylowanej kinazy białkowej regulowanej sygnałem pozakomórkowym 1/2 (fosfo-ERK1/2). Niezmieniony poziom markerów fuzji w mitochondriach komórek traktowanych Zol

i Ale, wraz ze zwiększonym poziomem markerów fragmentacji oraz białek mitochondrialnych, wskazują na utrzymywanie puli mitochondriów w obliczu zmniejszonego poziomu CoQ. W tej pracy po raz pierwszy pokazano, iż hamowanie szlaku mewalonowego przez N-BPs i tym samym obniżenie prenylacji białek może mieć wpływ na dynamikę mitochondriów.

Badania zużycia tlenu przez całe komórki śródbłonka poddane działaniu Zol lub Ale wykazały liczne adaptacje dotyczące metabolizmu tlenowego. W warunkach rozprzęgających, po podaniu glukozy lub palmitynianu do płynu pomiarowego, komórki traktowane N-BPs wykazywały zwiększoną szybkość zużycia tlenu (ang. *oxygen consumption rate* – OCR). Zarówno oddychanie bazowe jak i związane z produkcją ATP przy utlenianiu glukozy lub palmitynianu było wyższe w komórkach traktowanych Zol niż w komórkach kontrolnych. Jednocześnie, przy zastosowaniu substratów oddechowych takich jak glutamina, pirogronian czy mieszaniny wszystkich użytych w doświadczeniu substratów oddechowych (tj. glukoza, palmitynian, glutamina i pirogronian), zauważono ~20-35% spadek maksymalnej pojemności oddechowej mitochondriów (maksymalna OCR) oraz ~15-20% spadek OCR związanej z produkcją ATP. Co więcej, przy zastosowaniu mieszaniny wszystkich substratów oddechowych, zaobserwowano zwiększenie OCR niezwiązanej z produkcją ATP, świadczące o zwiększonym przecieku protonów. Najważniejsze wyniki uzyskane podczas tych doświadczeń wskazują na liczne adaptacje metabolizmu tlenowego komórek traktowanych N-BPs: zmniejszenie oddychania w komórkach traktowanych podczas utleniania „silniejszych" substratów (tj. glutaminy lub pirogronianu), zmniejszenie oddychania związanego z produkcją ATP czy zwiększenie przecieku protonów podczas utleniania mieszaniny substratów.

Permeabilizacja komórek śródbłonka za pomocą digitoniny umożliwiła badanie aktywności oddechowej mitochondriów przy zaangażowaniu poszczególnych dehydrogenaz substratowych. W permeabilizowanych komórkach traktowanych N-BPs oddychających w warunkach fosforylujących, zaobserwowano spadek utleniania bursztynianu podanego wraz z rotenonem (określenie aktywności kompleksu II przy jednoczesnym hamowaniu kompleksu I) na poziomie 11-15%, podczas gdy zmniejszenie szybkości zużycia tlenu podczas utleniania jabłczanu (substratu kompleksu I) wyniosło 22-30%. W permeabilizowanych komórkach śródbłonka poddanych działaniu N-BPs, współczynnik kontroli oddechowej (ang. *respiratory control ratio* - RCR) przy braku oligomycyny był znacząco zmniejszony po podaniu jabłczanu. W obecności oligomycyny RCR (RCR_{oligo}) był zmniejszony zarówno podczas utleniania jabłczanu (substratu kompleksu I) jak i bursztynianu (substratu kompleksu II). Opisane wyniki wskazują na zwiększone rozprzęgnięcie mitochondriów komórek traktowanych N-BPs, co

może wpływać na zmniejszenie wydajności fosforylacji oksydacyjnej, a także na dysfunkcję łańcucha oddechowego, szczególnie podczas utleniania jabłczanu.

Podsumowując, badania przeprowadzone na komórkach śródbłonka poddanych 6-dniowemu działaniu Zol lub Ale wskazują na adaptację metabolizmu tlenowego tych komórek w odpowiedzi na działanie N-BPs. Badania te pokazują po raz pierwszy obniżenie komórkowego poziomu CoQ w wyniku hamowania szlaku mewalonowego przez N-BPs. Zmiany stanu redoks CoQ wywołane tymi lekami wpływają na funkcjonowanie mitochondriów, stan energetyczny w komórkach śródbłonka czy biogenezę i dynamikę mitochondriów. A zatem, N-BPs prowadzą do przeprogramowania metabolicznego komórek śródbłonka w odpowiedzi na obniżenie poziomu CoQ, który jest ważnym przenośnikiem elektronów w mitochondrialnym łańcuchu oddechowym, a także ważnym komórkowym przeciwutleniaczem.

Publikacja 2: Budzinska A, Galganski L, Wojcicki K, Jarmuszkiewicz W, (2025), Adaptation of mitochondrial bioenergetic activity associated with coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates, alendronate and zoledronate, Scientific Reports, 15, (IF5y 4,3), doi: 10.1038/s41598-025-02710-8

Badania opisane w tej publikacji stanowią pogłębienie poprzednich badań, w których opisano wpływ N-BPs, wynikający z hamowania szlaku mewalonowego, na adaptacje metabolizmu tlenowego na poziomie całych komórek śródbłonka linii EA.hy926. Celem tej pracy było przyjrzenie się zmianom wywołanym przez 6-dniową hodowlę komórek śródbłonka EA.hy926 w obecności Zol lub Ale w ich metabolizmie tlenowym na poziomie mitochondrialnym i aktywności bioenergetycznej mitochondriów. W tym celu prowadzono badania na mitochondriach izolowanych z komórek śródbłonka traktowanych 5 µM Ale lub 1 µM Zol.

Do tej pory nie badano zmian poziomu mtCoQ wywołanego przez N-BPs. W pracy po raz pierwszy pokazano, że poziom całkowitego mtCoQ ($mtCoQH_2$ + mtCoQ utleniony) w mitochondriach komórek śródbłonka traktowanych N-BPs zmniejsza się o 45-50%. Dodatkowo zauważono ~20% spadek poziomu CoQ10B, białka niezbędnego do poprawnego funkcjonowania CoQ w łańcuchu oddechowym. Zaobserwowano także całkowitą utratę zredukowanej formy mtCoQ ($mtCoQH_2$) pełniącej funkcję przeciwutleniacza, która w komórkach kontrolnych stanowiła ~12% całej puli mtCoQ. Utracie $mtCoQH_2$ towarzyszył 10-20% wzrost poziomu dysmutazy ponadtlenkowej 2 (SOD2), co wskazuje na zwiększenie obrony antyoksydacyjnej w mitochondriach komórek traktowanych N-BPs.

Znaczne obniżenie przez N-BPs poziomu mtCoQ oznacza niedobór ważnego przenośnika elektronów w łańcuchu oddechowym. Następnym krokiem było zatem zbadanie utleniania różnych substratów oddechowych przez mitochondria izolowane z komórek traktowanych Ale lub Zol. Eksperymenty te pokazały, że w warunkach rozprzęgających, przy maksymalnej aktywności łańcucha oddechowego, szybkość zużycia tlenu podczas utleniania palmitylokarnityny rosła o ~20% w porównaniu z mitochondriami komórek kontrolnych. Towarzyszyło temu zwiększenie poziomu enzymu dehydrogenazy acetylo-CoA (ACADS), biorącej udział w początkowych etapach β-oksydacji. Obserwacje te wskazują na wzrost wykorzystania kwasów tłuszczowych jako substratów w metabolizmie tlenowym mitochondriów komórek traktowanych N-BPs. Aczkolwiek, w mitochondriach komórek

traktowanych N-BPs zaobserwowano obniżenie szybkości zużycia tlenu przy utlenianiu glutaminianu o ~13% w porównaniu do mitochondriów kontrolnych. Dodatkowo, poziom enzymu dehydrogenazy glutaminianowej (GDH), odpowiedzialnej za przekształcanie glutaminianu do α-ketoglutaranu, który włącza się do cyklu Krebsa, obniżył się o ~13%. Maksymalna szybkość zużycia tlenu podczas utleniania silnych redukujących substratów obniżała się o ~17% w przypadku jabłczanu (substratu kompleksu I), o ~25% w przypadku bursztynianu (substratu kompleksu II) i o ~21% przy użyciu mieszaniny obu substratów.

Oceniono również bioenergetyczne parametry mitochondriów śródbłonka z komórek kontrolnych i traktowanych N-BPs podczas utleniania bursztynianu (w obecności rotenonu), jabłczanu oraz mieszaniny obu substratów oddechowych w warunkach fosforylujących i niefosforylujących. Długotrwała, 6-dniowa ekspozycja komórek śródbłonka na Zol lub Ale spowodowała, że parametry sprzężenia mitochondriów tj. współczynnik ADP/O oraz współczynnik kontroli oddechowej były obniżone o 13-19%, co świadczy o zmniejszonej wydajności systemu fosforylacji oksydacyjnej. Szybkość fosforylacji ADP w mitochondriach komórek traktowanych N-BPs była niższa o ~30% przy użyciu jabłczanu jako substratu oddechowego, a przy użyciu bursztynianu o ~40%. Wyniki te wskazują na znacząco zmniejszoną syntezę ATP w mitochondriach komórek śródbłonka poddanych działaniu N-BPs.

Mitochondria komórek śródbłonka hodowane w obecności N-BPs wykazały mniejszą szybkość zużycia tlenu podczas utleniania jabłczanu (~20%) jedynie w warunkach fosforylujących w porównaniu z mitochondriami komórek kontrolnych. Co więcej, podczas utleniania bursztynianu, w warunkach niefosforylujących spadek szybkości zużycia tlenu wynosił ~17%, a w warunkach fosforylujących obniżenie wynosiło ~30%. Przy zastosowaniu mieszaniny obu substratów oddechowych szybkość oddychania mitochondriów obniżyła się o ~13% w warunkach niefosforylujących i ~26% w warunkach fosforylujących. W mitochondriach komórek traktowanych Zol lub Ale, obniżeniu utleniania jabłczanu i bursztynianu w warunkach niefosforylujących towarzyszyło obniżenie mitochondrialnego potencjału błonowego ($\Delta\Psi$), szczególnie widoczne podczas utleniania bursztynianu. W warunkach fosforylujących, jedynie przy zastosowaniu bursztynianu zaobserwowano spadek $\Delta\Psi$. Zatem, mitochondria komórek śródbłonka poddanych działaniu Zol lub Ale w porównaniu do mitochondriów pochodzących z komórek kontrolnych prezentują mniejszą szybkość oddychania, mniejszy $\Delta\Psi$ i obniżoną wydajność systemu fosforylacji oksydacyjnej. Opisane zmiany dotyczące funkcjonowania mitochondriów komórek traktowanych N-BPs były szczególnie wyraźne podczas utlenianiu bursztynianu w porównaniu do utleniania jabłczanu.

W mitochondriach komórek śródbłonka poddanych działaniu N-BPs produkcja H_2O_2 była wyższa w porównaniu z mitochondriami komórek kontrolnych w warunkach fosforylujących i niefosforylujących niezależnie od użytego substratu oddechowego. Produkcja H_2O_2 była szczególnie zwiększona podczas utleniania jabłczanu (~22-25%) w porównaniu do utleniania bursztynianu (~13-15%). Ponadto, w mitochondriach komórek traktowanych N-BPs poziom redukcji mtCoQ ($mtCoQH_2$/mtCoQ całkowity) rósł o ~18% z jabłczanem i ~13% z bursztynianem. A zatem, w mitochondriach komórek śródbłonka traktowanych Zol lub Ale, wzrost produkcji mtRFT był związany z zwiększonym poziomem redukcji mtCoQ, wynikającym ze zmniejszonej puli mtCoQ.

W mitochondriach komórek kontrolnych i traktowanych N-BPs, w warunkach fosforylujących i niefosforylujacych podczas utleniania jablczanu i/lub bursztynianu, potwierdzono bezpośrednią zależność produkcji mitochondrialnych RFT (mtRFT) od poziomu redukcji mtCoQ. Wykres zależności produkcji mtRFT od $\Delta\Psi$ dla mitochondriów komórek poddanych działaniu N-BPs był przesunięty w stosunku do wykresu dla mitochondriów komórek kontrolnych w kierunku większych wartości produkcji H_2O_2 i niższych wartości $\Delta\Psi$, wskazując na hamowanie ścieżki utleniającej $mtCoQH_2$. Dodatkowo, aktywność kompleksu III była niższa w mitochondriach komórek traktowanych o ~20%, podczas gdy aktywność kompleksu IV pozostała taka sama. Zauważono również ~17% spadek redukcji cytochromów $a+a_3$ (wchodzących w skład kompleksu IV), co wskazuje na obniżenie poziomu hemów *a*, będących obok CoQ produktami szlaku mewalonowego hamowanego przez N-BPs.

Aby zrozumieć, dlaczego w mitochondriach komórek śródbłonka traktowanych N-BPs, utlenianie substratu kompleksu II powodowało silniejsze zmniejszenie oddychania i $\Delta\Psi$, ale słabszy wzrost produkcji mtRFT i poziomu redukcji mtCoQ - w porównaniu z utlenianiem substratu kompleksu I - zbadaliśmy zmiany w poszczególnych składnikach systemu fosforylacji oksydacyjnej oraz ich molekularnej organizacji. Analiza poziomów kompleksów łańcucha oddechowego pokazała ~20% spadek poziomu podjednostki SDHB (składowej kompleksu II), poziomu podjednostki Core 2 (składowej kompleksu III, CIII) i poziomu podjednostki α (składowej syntazy ATP) w mitochondriach komórek traktowanych N-BPs. Poziom podjednostki NDUFB8 (składowej kompleksu I, CI) i podjednostki COXII (składowej kompleksu IV, CIV) pozostał niezmieniony. Dalsze eksperymenty wykazały spadek aktywności kompleksu II i syntazy ATP w mitochondriach komórek traktowanych N-BPs. Aktywność kompleksu I we wszystkich jego superkompleksach pozostała niezmieniona. Poziom superkompleksów obejmujących CI + CIII ($I + III_2 + IV_{(n)}$ oraz $I + III_2$) nie zmieniał

się w mitochondriach komórek traktowanych N-BPs. Natomiast poziom superkompleksu III_2 + IV i dimeru III_2 zmniejszył się. Podczas badania aktywności enzymów w żelu zaobserwowano obniżenie aktywność kompleksu IV tylko w superkompleksie III_2 + IV. Aktywność innych superkompleksów/kompleksów związanych z kompleksem IV oraz poziom podjednostki COXII, wchodzącej w skład tego kompleksu, były niezmienione. Opisane zmiany oraz reorganizacja molekularnej struktury superkomplesów łańcucha oddechowego mogą być istotne dla efektywniejszego wykorzystania zmniejszonej puli mtCoQ wywołanej działaniem N-BPs.

Następnie zbadano czy zwiększona produkcja mtRFT w mitochondriach komórek traktowanych N-BPs wpływa na mitochondrialne systemy rozpraszające energię tj. białka rozprzęgające (UCP) oraz mitochondrialny kanał potasowy o dużym przewodnictwie regulowany Ca^{2+} ($mitoBK_{Ca}$). W mitochondriach komórek traktowanych N-BPs poziom UCP2 był o ~27% wyższy, podczas gdy poziom UCP3 nie uległ zmianie. Co więcej, poziom podjednostek kanału $mitoBK_{Ca}$, podjednostki porowej α i podjednostki regulacyjnej sloβ2, był niezmieniony. Badania aktywności obu systemów rozpraszających energię były zgodne z tymi obserwacjami. Nie obserwowano zmian w aktywności kanału $mitoBK_{Ca}$ w mitochondriach komórek traktowanych N-BPs w porównaniu do mitochondriów kontrolnych. Natomiast, pomiary kinetyczne wykazały zwiększoną o ~40% aktywność UCP indukowaną przez kwasy tłuszczowe i hamowaną przez GTP. Można przypuszczać, że zwiększona aktywność UCP wynikała ze zwiększonego poziomu UCP2. Podczas miareczkowania aktywności dehydrogenaz redukujących mtCoQ (kompleksów I i II), stopniowa depolaryzacja błony mitochondrialnej była związana z rosnącą wrażliwością aktywności UCP na GTP. Wyniki te potwierdzają, że niższy poziom redukcji mtCoQ prowadzi do większej inhibicji aktywności UCP przez nukleotydy purynowe. Wyniki te wskazują na to iż, w obliczu spadku poziomu mtCoQ i zwiększeniu produkcji mtRFT w mitochondriach komórek poddanych działaniu N-BPs, dochodzi do zwiększenia poziomu i aktywności UCP2, co może obniżać produkcję mtRFT w warunkach niedoboru mtCoQ.

Podsumowując, w komórkach śródbłonka poddanych działaniu Ale lub Zol, dochodzi do upośledzenia funkcjonowania mitochondrialnego łańcucha oddechowego oraz zmniejszenia wydajności fosforylacji oksydacyjnej. W mitochondriach komórek traktowanych N-BPs, zwiększony poziom redukcji mtCoQ (zwiększony stan redoks mtCoQ) prowadzi do zwiększenia produkcji mtRFT. N-BPs wywołują istotną reorganizację systemu fosforylacji oksydacyjnej oraz zwiększenie udziału utleniania kwasów tłuszczowych w mitochondriach.

Przedstawione badania stanowią pierwszy opis adaptacji bioenergetyki mitochondriów do niedoboru mtCoQ w komórkach poddanych długotrwałej ekspozycji na N-BPs.

Publikacja 3: Budzinska A, Jarmuszkiewicz W, (2025), Consequences of inhibition of mevalonate pathway by bisphosphonates at cellular and mitochondrial levels, manuskrypt wysłany (czerwiec 2025) do czasopisma *Pharmaceuticals*, w recenzji, zdeponowany w preprints.org, doi: 10.20944/preprints202506.2071.v1

Poniżej opisano najważniejsze punkty omawiane w pracy przeglądowej bez podawania pozycji literaturowych.

Praca zbiera dotychczasową wiedzę dotyczącą bisfosfonianów (BPs) i ich wpływu na funkcjonowanie komórek i ich mitochondriów. Najnowsze doniesienia literaturowe zebrane są w poszczególne rozdziały, od historii i podziału BPs, przez mechanizm działania aż do ich wpływu na poziomie komórkowym i mitochondrialnym. Na początku opisano osteoporozę jako jednostkę chorobową, w leczeniu której powszechnie stosowane są BPs. Duży popyt na leki antyresorpcyjne doprowadził do powstawania coraz nowszych formuł oraz generacji BPs, które są coraz silniejsze oraz skuteczniejsze w leczeniu osteoporozy.

Opis budowy, jak i mechanizmu działania poszczególnych typów BPs wyjaśnia istotne różnice między nimi. Przedstawiono dwie klasy BPs: starej generacji (niezawierające azotu) oraz nowszych generacji (zawierające azot - N-BPs). Odejście od BPs starej generacji na rzecz N-BPs wynika z faktu, iż te posiadające azot wykazują większe powinowactwo do kryształów hydroksyapatytu, który w większych ilościach występuje w miejscach o dużym tempie kościogubienia.

Następnie, opisano szlak mewalonowy, który blokują N-BPs. Podrozdział ten skupia się kolejno na każdym ważniejszym produkcie szlaku i jego roli. Poznanie skutków hamowania szlaku przez N-BPs ułatwia zrozumienie szerokiego zakresu efektów ubocznych ich działanie na różne układy i narządy. Szlak mewalonowy jest kluczowym nie tylko do osiągnięcia przez osteoklasty aktywności resorpcyjnej, ale również jest niezbędny do prawidłowego funkcjonowania szeregu innych komórek. Aczkolwiek, oprócz negatywnych skutków inhibicji tego szlaku, podkreślono również potencjał terapeutyczny użycia N-BPs w przypadku leczenia nowotworów. Jednocześnie wskazano na potrzebę dopracowania formuły N-BPs, która umożliwiałaby ominięcie wysokiego powinowactwa do kości i pozwalała na terapię celowaną.

Zebrana dostępna wiedza na temat działania N-BPs na komórki łączy skutki niedoboru produktów szlaku mewalonowego z mechanizmem działania N-BPs. Opisane efekty

hamowania szlaku wpływają na funkcjonowanie całego organizmu. Oprócz obniżenia poziomu ważnego antyoksydanta, CoQ, leki te zapobiegają prenylacji białek sygnałowych, takich jak Ras czy Rho, niezbędnych do transdukcji sygnałów w komórce. Biorąc pod uwagę poszczególne produkty szlaku mewalonowego i ich rolę w procesach komórkowych, opisano negatywne działanie N-BPs poprzez hamowanie szlaku mewalonowego w zdrowych komórkach, jednocześnie wskazując potencjał leczniczy N-BPs w terapii antynowotworowej.

Badania dotyczące komórek kości dostarczają najwięcej danych o tym jak działają na nie N-BPs. Ponieważ osteoklasty stanowią główny cel terapii antyresorpcyjnej, zbadano jak N-BPs działają nie tylko na nie, ale również na komórki nadbudowujące kość (osteoblasty) oraz na komórki różnicujące do osteoklastów. Pierwsze doniesienia o hamowaniu kanałów wapniowych przez N-BPs w komórkach prekursorowych osteoklastów otwiera nowy kierunek badania skutków ubocznych tych leków.

Kolejno opisano efekty obejmują wpływ N-BPs na komórki inne niż komórki kości. Porównywanie między sobą badań opartych na różnych liniach komórkowych z potencjalnym wpływem in vivo jest niemożliwe, ponieważ często stężenia użyte w tych badaniach przekraczają stężenia używane w terapiach chorób kostnych. Efekt antynowotworowy N-BPs udowodniony jest przez szereg opublikowanych prac, głównie skupiających się na zmniejszeniu prenylacji białek, takich jak Ras i Rho, poprzez hamowanie szlaku mewalonowego. Zmniejszona preneylacja tych białek negatywnie wpływa na polimeryzację cytoszkieletu aktynowego w komórkach nowotworowych ale także może mieć podobny wpływ na zdrowe komórki fibroblastów czy komórki mięśni gładkich naczyń krwionośnych.

N-BPs są wydalane z organizmu przez nerki. W związku z tym, powstało wiele badań oceniających potencjalną toksyczność tych związków dla komórek nerek, głównie poprzez wywoływanie stresu oksydacyjnego i apoptozy.

Poprzez wpływ na szlak sygnałowy EGFR/Akt/PI3K, N-BPs wywołują aktywację jądrowego czynnika NFκB, który stymuluje odpowiedź zapalną oraz apoptozę komórki. Oprócz wzrostu cytokin prozapalnych, N-BPs, poprzez zahamowanie prenylacji w wyniku blokowania szlaku mewalonowego, powodują zmniejszenie poziomu białek mających za zadanie niwelować odpowiedź zapalną.

W pracy opisano także najnowsze dane dotyczące efektów blokowania szlaku mewalonowego przez N-BPs związanych z obniżeniem poziomu CoQ w komórkach. Do niedawna, jedynie zaobserwowano obniżony poziom CoQ w surowicy kobiet w wieku

pomenopauzalnym przyjmujących N-BPs w ramach terapii antyresorpcyjnej. Publikacja naszej grupy opisuje po raz pierwszy zmiany metabolizmu tlenowego wywołane zmniejszonym poziomem CoQ w komórkach śródbłonka traktowanych N-BPs. Badania te podkreślają powiązanie obniżonego poziomu tego ważnego komórkowego przeciwutleniacza ze stresem oksydacyjnym. Wywołane przez N-BPs zmniejszenie zużycia tlenu przez komórki śródbłonka może prowadzić do zwiększonego poziomu tlenu, przyczyniając się do zwiększenia produkcji RFT i indukowania stresu oksydacyjnego.

Jak dotąd słabo poznano wpływ N-BPs na metabolizm lipidów. W wątrobie myszy karmionych dietą wysokotłuszczową, Zol zmniejszał poziom lipidów i zwiększał ekspresję genów związanych z utlenianiem kwasów tłuszczowych. W komórkach śródbłonka obserwowano wzrost utleniania palmitynianu po traktowaniu Zol lub Ale, co wskazuje na przesunięcie metabolizmu w kierunku katabolizmu kwasów tłuszczowych. Jednakże inne badania wykazały, że Zol może powodować akumulację kwasów tłuszczowych w komórkach nerkowych, co może prowadzić do nefrotoksyczności, włóknienia tkanek lub chorób nerek.

Zaburzenia autofagii są powiązane z silnym ograniczeniem prenylacji białek, co może prowadzić do aktywacji inflamasomu i śmierci komórki. Mimo że N-BPs, jako inhibitory szlaku mewalonowego, mogą wpływać na autofagię, ich działanie w komórkach innych niż kostne jest słabo poznane. Szlak mewalonowy wspiera prenylację małych GTPaz, m.in. białek Rab, istotnych dla kontroli jakości mitochondriów, w tym ich fragmentacji i mitofagii. Dotychczas tylko jedno badanie opisuje, że N-BPs mogą wpływać na dynamikę i obrót mitochondriów. W komórkach śródbłonka traktowanych N-BPs zauważono spadek poziomu markerów fragmentacji mitochondriów przy jednoczesnym braku zmian w poziomie markerów biogenezy i fuzji mitochondriów, sugerujące ograniczoną degradację mitochondriów przez mitofagię, prawdopodobnie w wyniku osłabienia sygnalizacji ERK1/2.

N-BPs mogą obniżać poziom wapnia we krwi poprzez hamowanie resorpcji kości, co zmniejsza uwalnianie wapnia do krwiobiegu. Obecnie istnieją ograniczone dowody na to, że N-BPs zaburzają gospodarkę wapniową w innych tkankach niż kości i krew. W jednym z badań wykazano, że Ale wpływa na wewnątrzkomórkową dynamikę wapnia w kardiomiocytach w warunkach in vitro.

Następny podrozdział omawia nieliczne badania dotyczące wpływu N-BPs na poziomie mitochondrialnym. Działanie N-BPs na poziomie mitochondrialnym może wynikać z zahamowania szlaku mewalonowego i obniżenia poziomu jego produktów istotnych dla

funkcjonowania mitochondriów, takich jak hemy *a* oraz mtCoQ, a także zaburzenia mitochondrialnej homeostazy wapniowej.

Wykorzystując izolowane mitochondria, zbadano mechanizm apoptozy wywołanej przez Zol w ludzkich komórkach chłoniaka grudkowego HF28RA. Badanie to pokazało, iż Zol powodował zwiększoną przepuszczalność błony mitochondrialnej, uwolnienie cytochromu *c* do cytoplazmy, aktywację kaspazy-3 i fragmentację DNA – charakterystyczne cechy apoptozy zależnej od mitochondriów. Co ciekawe, suplementacja geranylgeraniol (GGOH), przywracająca prenylację białek, chroniła komórki chłoniaka przed apoptozą, co podkreśla znaczenie prenylowanych białek w utrzymaniu integralności mitochondriów i przeżywalności komórek.

W literaturze nie ma wielu danych dotyczących wpływu N-BPs na gospodarkę wapniową mitochondriów. Tak na przykład, w izolowanych mitochondriach komórek nerkowych, zaobserwowano zmniejszone uwalnianie wapnia z mitochondriów in vitro oraz zwiększenie jego poziomu in vivo. Natomiast etidronian (BP niezawierający azotu) wykazał działanie ochronne na nerki w warunkach niedokrwienia, prawdopodobnie poprzez zwiększoną sekwestrację wapnia w mitochondriach.

Do tej pory niewiele wiadomo było na temat zmian poziomu hemów *a* (składników niezbędnych dla funkcjonowania kompleksu IV łańcucha oddechowego) w mitochondriach w wyniku działania N-BPs. W mitochondriach komórek śródbłonka traktowanych N-BPs zaobserwowano spadek cytochromów $a + a_3$, co wskazuje na zmniejszoną zawartość hemów *a*, choć aktywność enzymatyczna kompleksu IV pozostała niezmieniona.

Niewiele badań dotyczy wpływu N-BPs na funkcje oddechowe mitochondriów, jednak dostępne dane wskazują na ich negatywne działanie. W mitochondriach izolowanych z nerek szczurów traktowanych Zol stwierdzono spadek aktywności dehydrogenaz, depolaryzację błony mitochondrialnej, jej zwiększoną przepuszczalność i zmniejszenie poziomu ATP. W mitochondriach komórek śródbłonka poddanych działaniu N-BPs obserwowano obniżoną aktywność oddechową (z wyjątkiem zwiększonego utleniania kwasów tłuszczowych), mniejszy potencjał błonowy oraz niższą efektywność syntezy ATP. Dodatkowo wykazano reorganizację superkompleksów łańcucha oddechowego, co wskazuje na zaburzenie struktury i funkcjonowania łańcucha oddechowego przez N-BPs.

N-BPs, jako inhibitory szlaku mewalonianowego, mogą zaburzać syntezę CoQ, w tym mtCoQ – kluczowego przenośnika elektronów w łańcuchu oddechowym. Po raz pierwszy, na

przykładzie mitochondriów izolowanych z komórek śródbłonka traktowanych N-BPs wykazano, że Zol i Ale wywołują silny niedobór całkowitego mtCoQ i zanik jego zredukowanej formy ($CoQH_2$), pełniącej funkcję antyoksydacyjną. Towarzyszący tym zmianom wzrost ekspresji mitochondrialnych białek antyoksydacyjnych (SOD2, UCP2), nie zapobiega wzrostowi produkcji nadtlenku wodoru (H_2O_2), co wskazuje na zakłócenie równowagi redoks i nasilony stres oksydacyjny. Takie zaburzenia mogą uszkadzać mitochondria i zaburzać homeostazę komórkową, co może przyczyniać się do rozwoju chorób, m.in. sercowo-naczyniowych, neurodegeneracyjnych a także do przyspieszonego starzenia się organizmu.

Podsumowując, praca zbiera dotychczas dostępną wiedzę na temat efektów hamowania szlaku mewalonowego przez N-BPs na poziomie komórkowym i mitochondrialnym. W zależności od zastosowania tych leków, efekty te są pożądane, jak w przypadku terapii wspomagającej przy leczeniu nowotworów lub niepożądane jak podczas terapii antyresorpcyjnej pacjentów z osteoporozą. Biorąc pod uwagę proponowane użycie N-BPs przeciwko komórkom nowotworowym, zwrócono uwagę na rozwinięcie nowoczesnych rozwiązań formulacji leków, w sposób aby prezentowały jak najmniej skutków ubocznych. Jednocześnie podkreślono konieczność rozwinięcia badań nad wpływem N-BPs na komórki inne niż kości, do czego skłaniają badania potwierdzające ich negatywny wpływ na komórki śródbłonka czy nerek.

Na koniec pracy przeglądowej określono przyszłe kierunki badań, które powinny obejmować analizę dysfunkcji mitochondriów wywołanej przez N-BPs, ze szczególnym uwzględnieniem funkcjonowania łańcucha oddechowego oraz mechanizmów kontroli jakości mitochondriów. Badania te powinny dotyczyć tkanek i narządów, które są szczególnie narażone na efekty uboczne N-BPs, takie jak nerki, mięśnie szkieletowe, serce czy mózg. Szczególną uwagę należy poświęcić ocenie suplementacji CoQ jako potencjalnej strategii ochronnej mającej na celu złagodzenie wywołanych przez N-BPs uszkodzeń mitochondriów oraz zaburzeń w metabolizmie energetycznym komórki. Dalsze badania nad złożonym powiązaniem pomiędzy szlakiem mewalonowym, funkcjonowaniem mitochondriów a ogólną homeostazą komórkową będą istotne dla optymalizacji klinicznego zastosowania N-BPs i ograniczenia efektów ubocznych w tkankach niebędących celem terapii.

7. Podsumowanie wyników i wnioski

Wyniki pracy doktorskiej podkreślają rolę CoQ jako przeciwutleniacza ale również jako istotnego elementu mitochondrialnego łańcucha oddechowego. Publikacje 1 i 2 jako pierwsze wiążą hamowanie szlaku mewalonowego przez N-BPs z obniżonym poziomem CoQ, który może być jednym z czynników powodujących stres oksydacyjny i zmniejszenie żywotności komórek śródbłonka. Obniżenie poziomu całkowitego CoQ i utrata jego zredukowanej puli na poziomie komórkowym i mitochondrialnym zaburza homeostazę redoks CoQ co istotnie wpływa na wzrost produkcji RFT w komórkach traktowanych N-BPs. Zwiększeniu produkcji RFT nie zapobiega uruchomienie białek antyoksydacyjnych zarówno w cytozolu (SOD1 i GR) jak i mitochondriach (UCP2, SOD2). Na zwiększony w komórkach śródbłonka stres oksydacyjny wywołany przez N-BPs wskazują obniżony poziom HIF1α i kilkukrotnie podwyższony poziom KDM6A, białka markerowego poziomu tlenu.

W komórkach śródbłonka hodowla w obecności N-BPs powoduje hamowanie zależnej od prenylacji ścieżki sygnałowej ERK1/2 czemu towarzyszy zwiększenie odpowiedzi zapalnej (większej dla Zol niż Ale) oraz zmiana w dynamice mitochondriów. Pomimo zwiększenia poziomu mitochondrialnych białek markerowych (CS, COX, VDAC1), wskazujących na większą ilość mitochondriów, poziom markerów biogenezy mitochondriów (PGC1α i NRF2), pozostał bez zmian. Zmniejszenie poziomu białek markerowych fragmentacji (MFF i fosfoDRP1), przy niezmienionym poziomie białka markerowych fuzji (OPA1), wskazuje na adaptację sieci mitochondrialnej do zmienionych warunków metabolicznych wywołanych przez N-BPs. Adaptacja ta może obejmować obniżoną mitofagię na co wskazuje zahamowanie ścieżki ERK1/2, regulującej procesy dynamiki mitochondriów i zależną od mitochondriów apoptozę.

N-BPs istotnie zmieniają metabolizm tlenowy komórek śródbłonka zarówno na poziomie komórkowym, jak i mitochondrialnym. Pomimo zwiększonej pojemności oddechowej komórek (większej aktywności CS i COX), prawdopodobnie wynikającej z większej ilości mitochondriów, w komórkach traktowanych N-BPs maleje utlenianie silnych substratów oddechowych (pirogronianu i glutaminy). Co ciekawe, w komórkach traktowanych N-BPs i w izolowanych z nich mitochondriach zwiększeniu ulega utlenianie palmitynianu, co wskazuje na przekierowanie metabolizmu w kierunku utleniania kwasów tłuszczowych. Obniżeniu utleniania pozostałych substratów oddechowych na poziomie komórkowym towarzyszy obniżenie utleniania substratów kompleksów I i II w mitochondriach komórek traktowanych N-BPs. Podczas utleniania tych substratów, obniżonej szybkości zużycia tlenu i

wartości ΔΨ towarzyszy zwiększony poziom redukcji mtCoQ prowadzący do zwiększonej produkcji mtRFT. Obniżone mitochondrialne parametry sprzężeniowe (ADP/O i współczynnik kontroli oddechowej) oraz zmniejszony komórkowy poziom ATP wskazują na zmniejszoną przez N-BPs syntezę ATP i wydajność fosforylacji oksydacyjnej.

Wywołany przez N-BPs niedobór mtCoQ jest powiązany ze zmianą poziomu i aktywności kompleksów systemu fosforylacji oksydacyjnej i jego molekularnej organizacji. W mitochondriach komórek śródbłonka traktowanych N-BPs obserwowano obniżony poziom i aktywność kompleksów II, III i syntazy ATP oraz obniżony poziom superkompleksu III_2 + IV. Po raz pierwszy zaobserwowano także, że N-BPs mogą zmniejszać poziom hemów *a* czemu nie towarzyszy jednak obniżona maksymalna aktywność kompleksu IV.

Po raz pierwszy badano wpływ N-BPs na metabolizm energetyczny i aktywność bioenergetyczną mitochondriów w komórkach i izolowanych mitochondriach śródbłonka. Konsekwencje metaboliczne i energetyczne indukowane przez N-BPs, na poziomie komórkowym i mitochondrialnym w komórkach innych niż kostne, nie były dotąd szeroko badane, co opisuje praca przeglądowa (Publikacja 3).

Przedstawione rozprawie doktorskiej badania na komórkach śródbłonka pozwalają sformułować następujące wnioski końcowe:

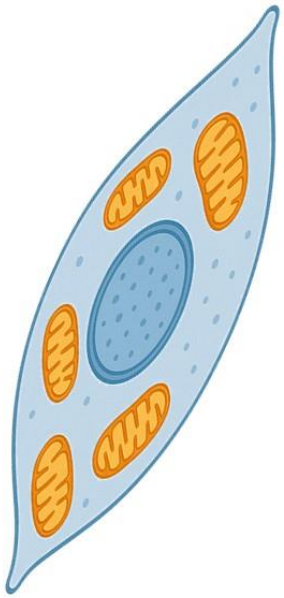
1. Badania stanowiące podstawę rozprawy doktorskiej podkreślają rolę mitochondriów w odpowiedzi komórek śródbłonka na N-BPs, powszechne leki antyresorpcyjne.
2. N-BPs wywołują adaptację metabolizmu tlenowego komórek śródbłonka obejmującą zmiany w utlenianiu substratów energetycznych.
3. Wywołane przez N-BPs zmiany w homeostazie redoks CoQ na poziomie komórkowym i mitochondrialnym prowadzą do zwiększonej produkcji RFT. Zmiany te obejmują zanik zredukowanej puli CoQ, pełniącej funkcję antyoksydacyjną, a także zwiększenie poziomu redukcji mtCoQ (stanu redoks mtCoQ), bezpośrednio związanego z produkcją mtRFT.
4. Zwiększona przez N-BPs produkcja RFT może być czynnikiem stymulującym adaptację metabolizmu tlenowego komórek śródbłonka towarzyszącą stresowi oksydacyjnemu.
5. Zahamowanie szlaku mewalonowego przez N-BPs, prowadzące do obniżenia poziomu CoQ i hemów *a* oraz do zmian w dynamice mitochondriów, może wywoływać dysfunkcję mitochondriów.

6. Obniżenie produkcji ATP i zaburzenie funkcjonowania łańcucha oddechowego mogą być istotnym elementem cytotoksycznego działania N-BPs na komórki śródbłonka.

Wpływ N-BPs na komórki śródbłonka

Poziom komórkowy:

- ↓ żywotność komórek
- ↑ stan zapalny
- ↓ szlak sygnalizacyjny ERK 1/2
- ↓ poziom ATP
- ↑ produkcja RFT
- ↓ poziom całkowitego CoQ, utrata $CoQH_2$
- Zmiana dynamiki mitochondriów: ↓ fizja, ↔ fuzja, ↑ ilość mitochondriów
- ↑ pojemność oddechowa
- Adaptacje metabolizmu tlenowego: ↑ utlenianie kw. tłuszczowych i glukozy ↓ utlenianie glutaminy i pirogronianu



Poziom mitochondrialny:

- ↓ poziom całkowitego mtCoQ, utrata $mtCoQH_2$
- ↓ utlenianie większości substratów oddechowych
- ↑ utlenianie kwasów tłuszczowych
- ↓ szybkość oddychania, ΔΨ, parametry sprzężenia
- ↑ produkcja mtRFT, poziom redukcji mtCoQ
- ↑ poziom UCP2, ↔ $mitoBK_{Ca}$
- ↑ SOD2
- ↓ poziom hemów *a*
- Zmiana aktywności i organizacji łańcucha oddechowego

Schemat podsumowujący wyniki opisane w publikacjach doświadczalnych składających się na rozprawę doktorską

8. Oświadczenia autora oraz współautorów publikacji składających się na rozprawę doktorską

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Oświadczenie określające wkład w powstanie artykułu

1. The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells (2023) *Scientific Reports* 13(1), DOI:10.1038/s41598-023-43377-3
   Budzinska A., Galganski L., Jarmuszkiewicz W.
   Udział doktorantki w badaniach opisanych w tej publikacji obejmował:
   - uczestniczenie w przygotowaniu koncepcji badań;
   - przygotowanie homogenatów i izolację mitochondriów;
   - przeprowadzenie pomiarów zużycia tlenu, ilości i poziomu redukcji koenzymu Q, produkcji RFT, poziomu ATP, aktywności enzymów;
   - uczestniczenie w immunodetekcji białek;
   - analizę danych, udział w przygotowaniu tekstu i rysunków manuskryptu.

2. Adaptation of mitochondrial bioenergetic activity associated with coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates, alendronate and zoledronate (2025) *Scientific Reports* 15, DOI: 10.1038/s41598-025-02710-8
   Budzinska A., Galganski L., Wojcicki K., Jarmuszkiewicz W.
   Udział doktorantki w badaniach opisanych w tej publikacji obejmował:
   - uczestniczenie w przygotowaniu koncepcji badań;
   - przygotowanie homogenatów i izolację mitochondriów;
   - przeprowadzenie pomiarów zużycia tlenu, potencjału błonowego, ilości i poziomu redukcji koenzymu Q, produkcji H_2O_2, redukcji cytochromu a + a_3;
   - uczestniczenie w immunodetekcji białek;
   - analizę danych, udział w przygotowaniu tekstu i rysunków manuskryptu;

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3. Consequences of inhibition of mevalonate pathway by bisphosphonates at cellular and mitochondrial levels, manuskrypt gotowy do wysłania
Budzinska A., Jarmuszkiewicz W.

Udział doktorantki w badaniach opisanych w tej publikacji obejmował:
- zebranie danych literaturowych i napisanie pierwszej wersji manuskryptu.

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Oświadczenie określające wkład w powstanie artykułu

Oświadczam, że mój wkład w powstanie wymienionych artykułów:

1. The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells (2023) *Scientific Reports* 13(1), DOI:10.1038/s41598-023-43377-3
   Budzinska A., Galganski L., Jarmuszkiewicz W.

2. Adaptation of mitochondrial bioenergetic activity associated with coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates, alendronate and zoledronate (2025) *Scientific Reports* 15, DOI: 10.1038/s41598-025-02710-8
   Budzinska A., Galganski L., Wojcicki K., Jarmuszkiewicz W.

polegał na uczestniczeniu w wykonaniu analizy immunoligicznej, BN-PAGE i pomiarze aktywności enzymów w żelu (in-gel activity) oraz opracowaniu wyników tych doświadczeń.

Łukasz Gałgański

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Poznań 30.05.2025

Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie artykułu: Budzinska A, Galganski L, Wojcicki K, Jarmuszkiewicz W, Adaptation of mitochondrial bioenergetics to coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates. *Scientific Reports* DOI : 10.1038/s41598-025-02710-8
polegał na uczestniczeniu w zbieraniu komórek i izolacji mitochondriów oraz pomocy w badaniach funkcjonalnych.

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Poznań, 16.06.2025

Oświadczenie określające wkład w powstanie artykułów

Oświadczam, że mój wkład w powstanie wymienionych artykułów polegał na:

1. The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells (2023) *Scientific Reports* 13(1), DOI:10.1038/s41598-023-43377-3
   Budzinska A., Galganski L., Jarmuszkiewicz W.

   - przygotowaniu koncepcji badań;
   - przygotowaniu manuskryptu;
   - opiece merytorycznej oraz finansowaniu badań poprzez kierowany przeze mnie projekt NCN.

2. Adaptation of mitochondrial bioenergetic activity associated with coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates, alendronate and zoledronate (2025) *Scientific Reports* 15, DOI: 10.1038/s41598-025-02710-8
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3. Consequences of inhibition of mevalonate pathway by bisphosphonates at cellular and mitochondrial levels, manuskrypt gotowy do wysłania
   Budzinska A., Jarmuszkiewicz W.

   - krytycznym czytaniu manuskryptu i jego przygotowaniu.

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The bisphosphonates alendronate and zoledronate induce adaptations of aerobic metabolism in permanent human endothelial cells

Adrianna Budzinska, Lukasz Galganski & Wieslawa Jarmuszkiewicz✉

Nitrogen-containing bisphosphonates (NBPs), compounds that are widely used in the treatment of bone disorders, may cause side effects related to endothelial dysfunction. The aim of our study was to investigate the effects of chronic 6-day exposure to two common bone-preserving drugs, alendronate and zoledronate, on endothelial function and oxidative metabolism of cultured human endothelial cells (EA.hy926). NBPs reduced cell viability, induced oxidative stress and a pro-inflammatory state and downregulated the prenylation-dependent ERK1/2 signaling pathway in endothelial cells. In addition, NBPs induced increased anaerobic respiration and slightly increased oxidative mitochondrial capacity, affecting mitochondrial turnover through reduced mitochondrial fission. Moreover, by blocking the mevalonate pathway, NBPs caused a significant decrease in the level of coenzyme Q10, thereby depriving endothelial cells of an important antioxidant and mitochondrial electron carrier. This resulted in increased formation of reactive oxygen species (ROS), upregulation of antioxidant enzymes, and impairment of mitochondrial respiratory function. A general decrease in mitochondrial respiration occurred with stronger reducing fuels (pyruvate and glutamate) in NBP-treated intact endothelial cells, and significantly reduced phosphorylating respiration was observed during the oxidation of succinate and especially malate in NBP-treated permeabilized endothelial cells. The observed changes in oxidative metabolism caused a decrease in ATP levels and an increase in oxygen levels in NBP-treated cells. Thus, NBPs modulate the energy metabolism of endothelial cells, leading to alterations in the cellular energy state, coenzyme Q10 redox balance, mitochondrial respiratory function, and mitochondrial turnover.

Bisphosphonates (BPs), the synthetic analogs of pyrophosphate, are among the most commonly prescribed drugs worldwide due to their effectiveness in the treatment of osteoporosis, other less common bone pathologies, and certain bone cancers[1,2]. BPs inhibit bone resorption by impeding osteoclast activity or by inducing apoptosis[3]. Nitrogen-containing bisphosphonates (NBPs) inhibit farnesyl diphosphate synthase (FPPS), a key enzyme of the intracellular mevalonate pathway, thereby preventing prenylation and activation of the small GTPases that are essential for bone resorption and osteoclast survival. The BP-inhibited mevalonate pathway is also responsible for the biosynthesis of cholesterol, other sterols, isoprenoid lipids, heme *a*, coenzyme Q[4,5].

Coenzyme Q is a key electron carrier in the mitochondrial respiratory chain and an important antioxidant that is present in all cell membranes[6]. In addition, it is involved in the production of mitochondrial reactive oxygen species (ROS) through the mitochondrial respiratory chain. A decrease in coenzyme Q levels, resulting from the blockage of the mevalonate pathway, may result in abnormal mitochondrial respiratory function, leading to oxidative damage[6,7]. Currently, there are few studies of the effects of NBPs on cell coenzyme Q10 levels. For example, NBP therapy has been associated with impaired coenzyme Q10 status in the plasma of postmenopausal women[8].

Endothelial cells line all blood vessels, and thus they are among the first cells that contact drugs such as BPs that are transported by the blood. Therefore, endothelial oxidative metabolism may be susceptible to changes

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in blood components, thereby contributing to oxidative stress. Endothelial dysfunction is closely related to the excessive production of ROS, including those in the mitochondria, and therefore may lead to the development of civilization diseases of the cardiovascular system[9–12].

Although the skeletal system is the primary target of BPs, studies have shown that these anti-osteoporosis drugs also affect the function of endothelial cells; the latter play key roles in vascular metabolism and homeostasis[13]. Prolonged or high doses of NBPs can cause adverse effects, including an increased risk of cardiovascular events or BP-related osteonecrosis of the jaw (BRONJ) in association with inhibition of angiogenesis[14–16]. NBPs, especially zoledronate, have been demonstrated to affect cell viability, cell migration, and apoptosis of human umbilical vein endothelial cells (HUVECs)[17]. In addition, NBPs negatively affect angiogenesis by inhibiting adhesion, proliferation, survival, migration, and formation of actin stress filaments in HUVECs by interfering with protein prenylation[18–21].

The effect of NBPs on the oxidative metabolism of endothelial cells, a process related to the level of coenzyme Q10, has not yet been characterized. Understanding the mechanisms by which NBPs affect the energy metabolism of endothelial cells is important to elucidate their potential impact on the proper functioning of the cardiovascular system.

The aim of our study was to investigate the effects of chronic 6-day exposure to two common bone-preserving NBPs, alendronate and zoledronate, on the endothelial function and aerobic metabolism of cultured human endothelial EA.hy926 cells. Coenzyme Q10 content, ROS production, ATP level and mitochondrial respiratory function were studied in control and bisphosphonate-treated cells. In addition, we examined the effects of NBPs on cell viability, markers of inflammation and oxygen level, and mitochondrial turnover.

Results

Dose-dependent effects of NBPs on cell viability and cellular coenzyme Q10 content

We selected two representative NBPs for the study: zoledronate, a potent bisphosphonate due to its unique R2 side chain consisting of a heterocyclic ring containing two nitrogen atoms, and alendronate, an NBP that has medium-level potency[22]. The micromolar concentrations used in the experiments were selected from a wide range of published studies on the concentrations of these compounds found in the serum of patients treated for osteoporosis or bone cancer after drug infusions[23–25].

Our initial goal was to select doses of alendronate and zoledronate that significantly affected the cellular Q10 content without impacting endothelial cell viability. We studied the effects of NBPs on endothelial cell viability using concentrations of 1–10 μM for alendronate and 0.5–5 μM for zoledronate (Fig. 1a). Higher concentrations (from 7.5 μM for alendronate and 2.5 μM for zoledronate) significantly reduced the viability of endothelial cells (Fig. 1a) and the content of Q10 (by ~ 60% for 7.5 μM alendronate and 2.5 μM zoledronate) in these cells (Fig. 1b). Therefore, subsequent experiments were performed with endothelial cells cultured for six days under controlled conditions without NBPs and with 5 μM alendronate or 1 μM zoledronate, concentrations that did not affect cell viability but significantly (by ~ 30%) reduced total cellular Q10 content.

Our results revealed that chronic exposure of endothelial cells to high concentrations of NBPs decreased cell viability, indicating the cellular toxicity of these compounds. In addition, we have demonstrated for the first time that NBPs significantly reduce cellular Q10 levels and that a severe deficiency of Q10 (~ 60%) may contribute to the loss of cell viability.

NBP-induced decrease in Q10 levels led to increased ROS formation and upregulation of antioxidant enzymes

The ~ 30% NBP-induced decrease in total cellular Q10 content was accompanied by elimination of the reduced Q10 ($Q10H_2$) pool that accounted for ~ 8% of the total Q10 (Q10 plus $Q10H_2$) pool in untreated cells (Fig. 2a). In endothelial cells treated with NBPs, a decrease in Q10, especially its reduced pool that functions as an antioxidant,

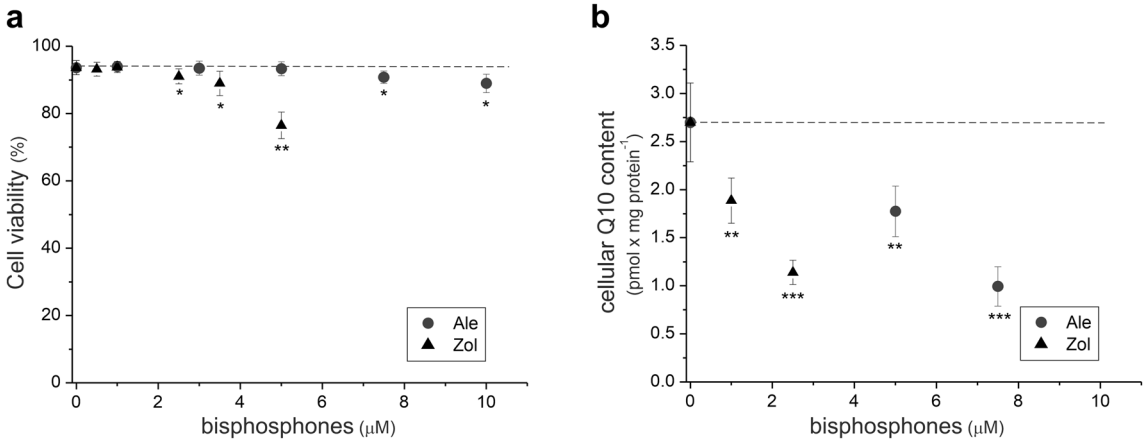

Figure 1. Dose-dependent effects of NBPs on viability (**a**) and cellular Q10 content (**b**) of endothelial cells (EA.hy926). Mean ± SD; $n = 5$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), comparison *vs.* control cells (Ctr). Ale, alendronate; Zol, zoledronate.

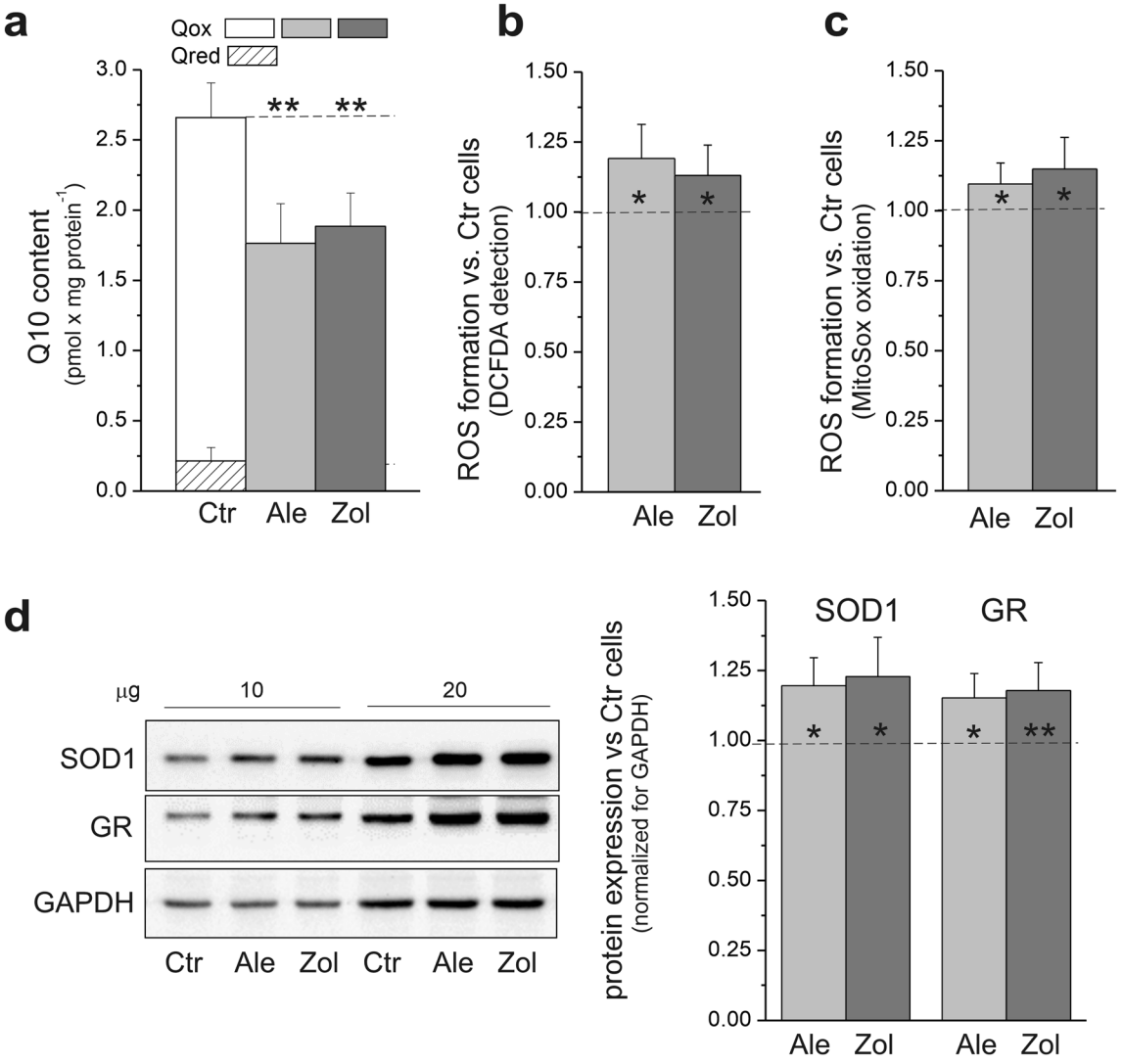

Figure 2. Effect of 5 µM alendronate (Ale) and 1 µM zoledronate (Zol) on total, oxidized, and reduced Q10 pool (**a**), total (**b**) and mitochondrial (**c**) ROS production and expression of antioxidant enzymes (**d**) in endothelial cells. **d**, Representative western blots (cropped blots) and analysis of protein expression are presented. Mean ± SD; $n = 8$. $P < 0.05$ (*), $P < 0.01$ (**), comparison *vs.* control cells (Ctr). SOD1, superoxide dismutase; GR, glutathione reductase.

led to increases in total cellular and mitochondrial ROS production (Fig. 2b and c). The additional ROS resulted in oxidative stress, as indicated by a 15–20% increase in the expression of the antioxidant enzymes glutathione reductase and superoxide dismutase 1 (Fig. 2d). The effects of 5 µM alendronate and 1 µM zoledronate were similar.

Our results indicated that in endothelial cells treated with NBPs, the greatly decreased Q10 level that led to oxidative stress was compensated for by increases in the other antioxidants (i.e., glutathione reductase and superoxide dismutase 1).

In endothelial cells, zoledronate significantly increased inflammation markers, while both tested NBPs increased oxygen level marker

Intercellular adhesion molecule 1 (ICAM1) is an adhesion receptor that regulates the recruitment of leukocytes from the circulation to endothelial cells at sites of inflammation[26,27]. The cytokine interleukin-6 (IL6) plays a key role in inflammation and directly affects vascular endothelial cells, which produce several types of cytokines and chemokines and activate the coagulation cascade[28]. In our study, increases in the expression of these inflammatory marker proteins were observed in cells treated with zoledronate, but not in alendronate-treated or untreated cells (Fig. 3). However, the applied concentrations of the NBPs (5 µM alendronate and 1 µM zoledronate) had no significant effect on the viability of endothelial cells (Fig. 1a).

NBP-treated cells showed significant (~ 20%) reductions in hypoxia-inducible factor 1α (HIF1α), which is a marker of hypoxia, and a six-fold increase in lysine (K)-specific histone demethylase 6A (KDM6A) that functions as a direct cellular oxygen sensor that regulates gene transcription. Oxygen levels via HIF1α regulate KDM6A to

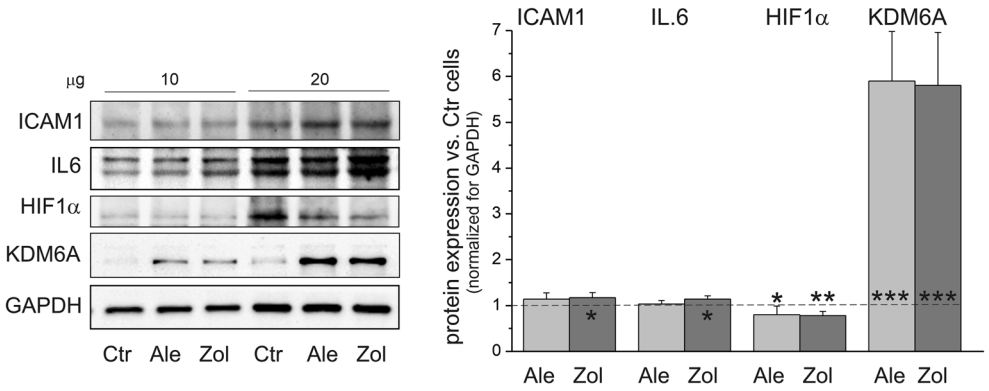

Figure 3. Effect of 6-day endothelial cell culture with 5 µM alendronate (Ale) and 1 µM zoledronate (Zol) on inflammation and oxygen level markers. Representative western blots (cropped blots) and analysis of protein expression are presented. Mean ± SD; $n = 6$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), comparison *vs.* control cells (Ctr). ICAM1, intercellular adhesion molecule 1; IL6, interleukin-6; HIF1α, hypoxia-inducible factor 1α; H KDM6A, lysine (K)-specific demethylase 6A.

control chromatin and cell fate[29,30]. Our results indicate elevated oxygen levels in NBP-treated cells, which may contribute to oxidative stress and changes in cellular oxidative metabolism.

In endothelial cells, NBPs upregulated mitochondrial oxidative capacities and anaerobic respiration

Citrate synthase (CS), the first enzyme of the tricarboxylic acid cycle (TCA cycle), and cytochrome *c* oxidase (COX), complex IV of the respiratory chain, are markers of mitochondrial oxidative function. Compared to control cells, endothelial cells treated with NBPs showed slight (~ 12–17%) increases in the expression levels (Fig. 4a) and activities (Fig. 4b and c) of CS and COX, indicating greater oxidative capacities of the TCA cycle and respiratory chain. The increased mitochondrial oxidative capacities were accompanied by a 27% increase in the expression level of hexokinase-1 (HK1), a key enzyme of glycolysis (Fig. 4a), and a significantly greater expression level (~ 16–20%) and activity (~ 25–35%) of lactate dehydrogenase (LDH) (Fig. 4a and d), an enzyme that converts pyruvate to lactate to maintain increased flux through glycolysis.

Thus, our results indicate that in endothelial cells, NBPs increase the oxidative capacity of mitochondria as well as anaerobic respiration. Despite the increase in respiratory capacity, a decrease (statistically significant in zoledronate-treated cells) in ATP levels was observed in the NBP-treated cells compared to untreated cells (Fig. 4e).

NBPs affected mitochondrial turnover by reducing mitochondrial fission in endothelial cells

The greater (up to 17%) levels of mitochondrial marker (CS and COX) activity and expression (Fig. 4) indicates an increased content of mitochondria in endothelial cells treated with NBPs. For example, in human skeletal muscle, CS activity in particular has shown a strong association with mitochondrial content[31]. In our study, increased mitochondrial content in NBP-treated endothelial cells was confirmed by a small (~ 10–15%) increase in the expression of the voltage-dependent anion-selective channel protein 1 (VDAC1) and mitochondrial non-glycosylated protein (MT), another mitochondrial markers (Fig. 5a). Therefore, we have checked NBP-induced changes in mitochondrial turnover markers, including those related to mitochondrial biogenesis and fission/fusion.

Despite the increase in oxidative stress in NBP-treated endothelial cells (increased ROS production) (Fig. 2b and c), no increases in the expression of mitochondrial biogenesis markers, i.e., peroxisome proliferator-activated receptor γ coactivator (PGC1α) and nuclear factor erythroid 2-related factor (NRF2), were observed (Fig. 5a). However, statistically significant decreases in the expression of fission markers, i.e., mitochondrial cleavage factor, MFF and active phospho-dynamin related protein 1, phospho-DRP1 (Ser616) (~ 25% and ~ 12%, respectively), combined with stable levels of a fusion marker (arthrosis protein-1, OPA1) indicated an NBP-induced change in mitochondrial turnover (Fig. 5). Since extracellular signal regulated protein kinase 1/2 (ERK1/2) has previously been shown to prevent inflammatory signaling[32] as well as regulate DRP1-dependent mitochondrial fusion[33] in endothelial cells, we also examined the expression levels of active phospho-ERK1/2 (Thr202/Tyr204) in NBP-treated endothelial cells. Figure 5b shows that significantly reduced levels (~ 36%) of phospho-ERK1/2 were observed in endothelial cells treated with NBPs compared to control cells, indicating downregulation of the ERK1/2 signaling pathway.

Our results revealed (i) slightly increased mitochondrial content, (ii) unaltered PGC1α/NRF2 mitochondrial biogenesis signaling pathway, (iii) reduced mitochondrial fission markers accompanied by unchanged fusion marker, and (iv) decreased ERK1/2 signaling pathway favoring the maintenance of the mitochondrial pool when there is a strong decrease in the level of Q10, which, in addition to antioxidant functions, is an important electron carrier in the mitochondrial respiratory chain.

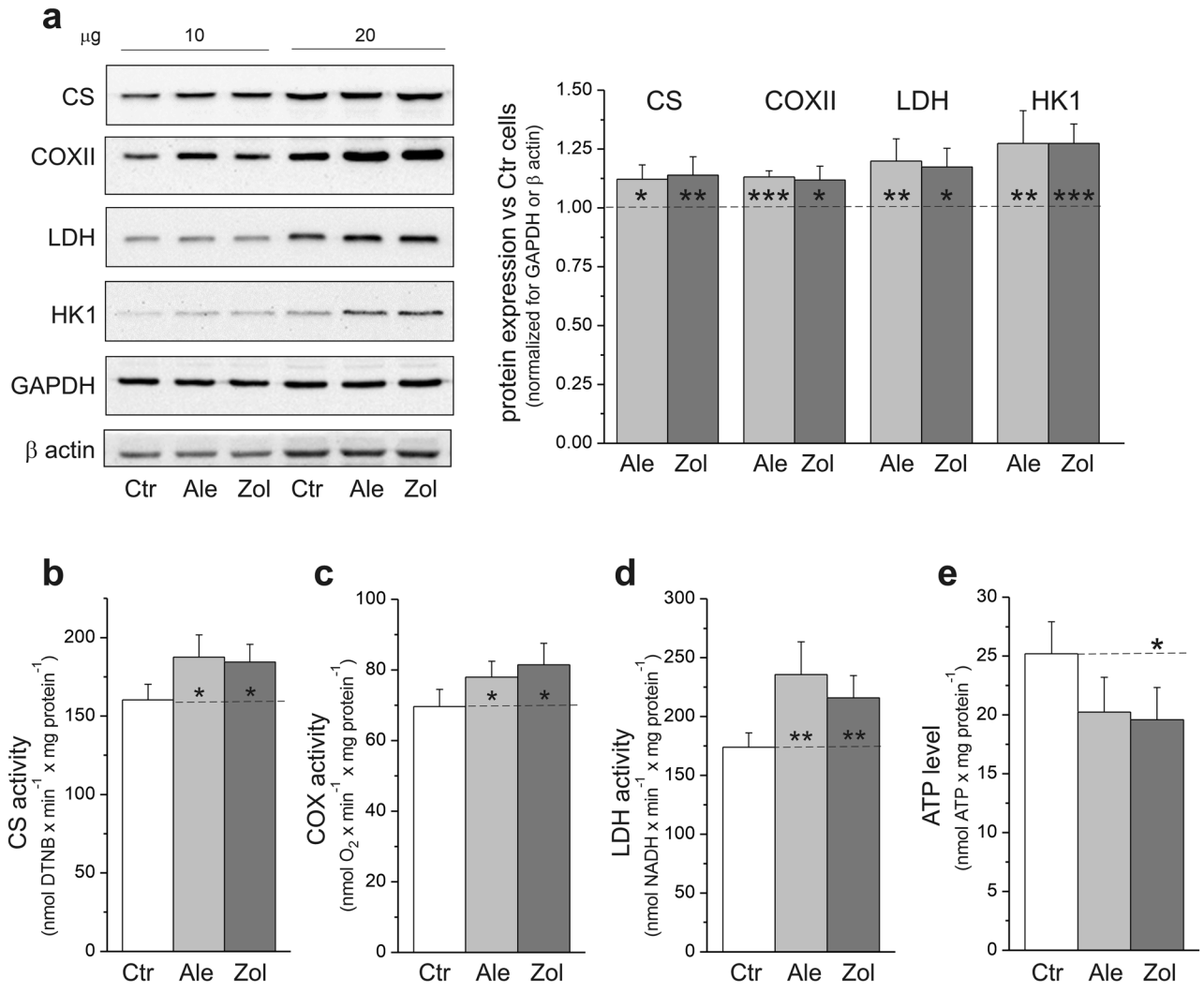

Figure 4. Effect of 6-day endothelial cell culture with 5 µM alendronate (Ale) and 1 µM zoledronate (Zol) on markers of mitochondrial and anaerobic respiration. Representative western blots (cropped blots) and analysis of protein expression (**a**). Protein expression levels were normalized for β actin (lactate dehydrogenase, LDH) or GAPDH (other proteins). Maximal aerobic (**b**, **c**) and anaerobic respiration (**d**) marker activities and ATP level (**e**). Mean ± SD; $n = 6–7$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), comparison vs. control cells (Ctr). CS, citric synthase; COXII, cytochrome *c* oxidase; HK1, hexokinase I.

In endothelial cells, NBPs induced an overall decrease in mitochondrial respiration except for the weak substrates glucose and palmitate

We next analyzed changes in the aerobic metabolism of endothelial cells cultured with 1 µM zoledronate or 5 µM alendronate by measuring the oxidation of different reducing substrates. NBP-treated cells showed increased respiration under uncoupling conditions (maximal oxygen consumption rate, OCR) with comparatively weaker substrates (glucose and palmitate) (Fig. 6). During the oxidation of these substrates, basal OCR and ATP-linked OCR showed statistically significant increases over control cells only in zoledronate treated cells.

However, for more potent respiratory substrates (glutamine, pyruvate and a mixture of all substrates tested), NBP-exposed cells displayed decreased respiration, indicating impairment of mitochondrial respiratory function. For these substrates, NBP-treated cells showed ~ 20–35% reductions in maximal mitochondrial respiratory capacity (maximal OCR) (Fig. 6b) as well as ~ 15–20% decreases in ATP-linked OCR (statistically insignificant for glutamine in alendronate-treated cells) (Fig. 6d), indicating reduced mitochondrial oxidative phosphorylation. In addition, during the oxidation of the mixture of all substrates, an increase in non-ATP-linked OCR (proton leak) was observed (Fig. 6c).

Thus, in NBP-treated cells, we observed (i) a decrease in mitochondrial respiration when strong respiratory substrates pyruvate and glutamine were oxidized alone or mixed, (ii) an increase in proton leak during the oxidation of a mixture of all substrates, and (iii) a decrease in ATP-related respiration with stronger substrates, indicating significant changes in energy metabolism.

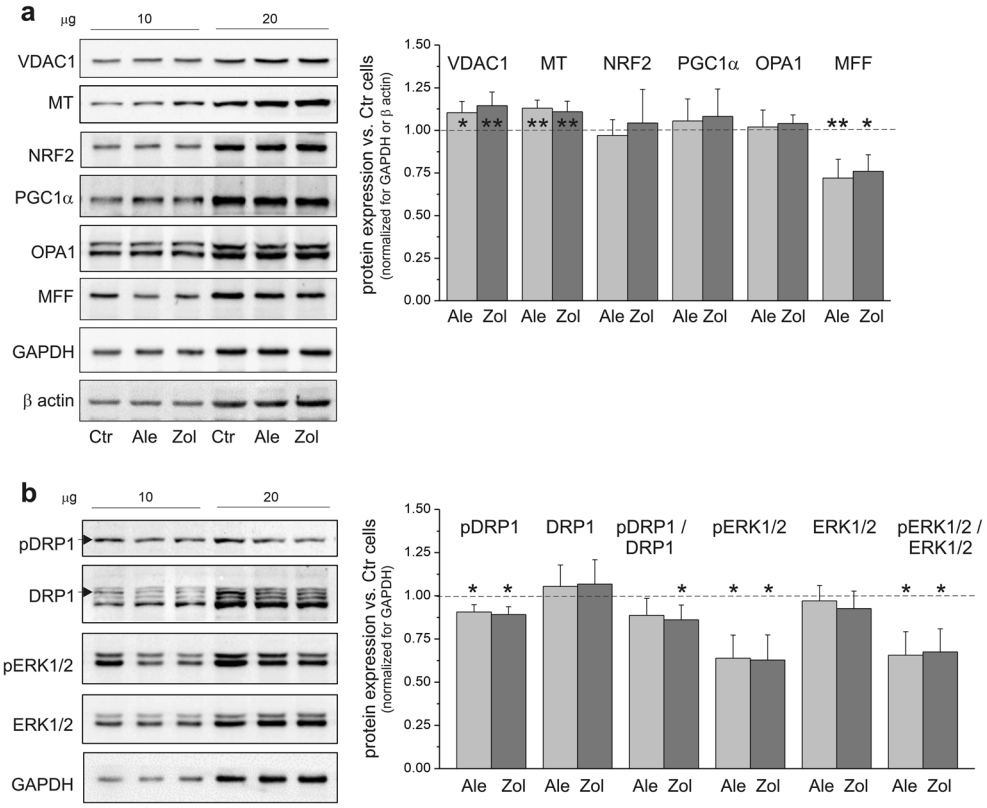

Figure 5. Markers of mitochondrial dynamics of endothelial cells grown with 5 μM alendronate (Ale) and 1 μM zoledronate (Zol). Representative western blots (cropped blots) and analysis of protein expression are presented. Mean ± SD; $n = 6$. $P < 0.05$ (*), $P < 0.01$ (**), comparison *vs.* control cells (Ctr). (**a**) VDAC1, voltage-dependent anion-selective channel protein 1; MT, mitochondrial marker, mitochondrial non-glycosylated protein; NRF2, nuclear factor erythroid 2-related factor, PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; OPA1, artrophy-1 protein; MFF, mitochondrial cleavage factor. (**b**) pDRP1, phospho-dynamin related protein 1; DRP1, total DRP1 (pDRP1 marked with an arrow); pERK1/2, phospho-extracellular signal-regulated protein kinase 1/2; ERK1/2, total ERK1/2. Protein expression levels were normalized for β actin (MFF) or GAPDH (other proteins).

Permeabilized NBP-treated endothelial cells showed decreased phosphorylating respiration and respiratory control ratios in the presence of oligomycin. The effect was more pronounced during complex I substrate oxidation compared to complex II substrate oxidation

Measurement of OCR in permeabilized cells allows for the respiratory activity of mitochondria with complex II (succinate) and complex I (malate) substrates to be determined. In our study, succinate oxidation (complex II activity) was measured in the presence of rotenone to inhibit complex I activity. Permeabilized endothelial cells treated with NBPs showed reduced phosphorylating respiration with both succinate (by 11–15%) and malate (by 22–30%) (Table 1). A reduction in nonphosphorylating respiration was also observed (statistically significant for succinate). Interestingly, in permeabilized NBP-treated endothelial cells during both succinate and malate oxidation, nonphosphorylating respiration was higher (by 11–20%) in the presence of oligomycin, which inhibits ATP conversion. This result indicated increased mitochondrial respiration that was unrelated to ATP synthesis in cells cultured with NBPs, similar to such respiration measured with intact cells (Fig. 6c). However, the level of mitochondrial uncoupling protein 2 (UCP2) was unchanged in NBP-treated cells compared to control cells (Supplementary Fig. S1). In permeabilized NBP-treated endothelial cells, the respiratory control ratio in the absence of oligomycin (RCR) was significantly reduced with malate, while that in the presence of oligomycin (RCR_{Oligo}) was decreased for both substrates. RCR_{Oligo} represents the maximal factorial increase in mitochondrial OCR induced by the phosphorylation of ADP to ATP that can be achieved above the proton leak driven OCR when ADP recycling is blocked by oligomycin.

Thus, our results indicated that endothelial cells cultured with NBPs had (i) greater mitochondrial uncoupling and thus reduced oxidative phosphorylation efficiency, and (ii) respiratory chain inhibition, especially during oxidation of the complex I substrate.

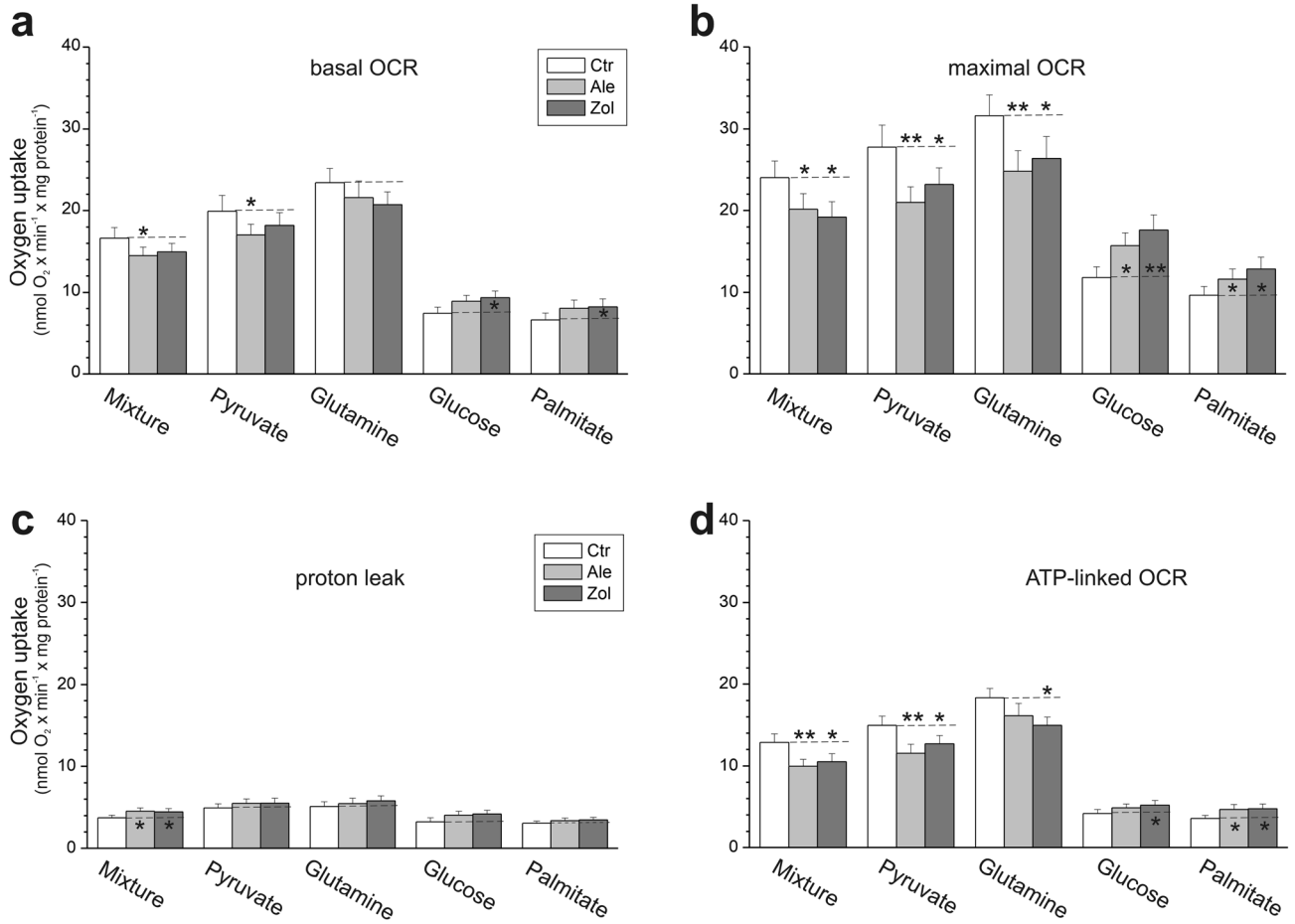

Figure 6. Effect of endothelial cell culture with 5 µM alendronate (Ale) and 1 µM zoledronate (Zol) on mitochondrial oxidative metabolism. Changes in the basal oxygen consumption rate (OCR) (**a**), maximal OCR (**b**), proton leak (**c**), and ATP-linked OCR (**d**) using different reducing substrates. Mean ± SD; $n = 6$. $P < 0.05$ (*), $P < 0.01$ (**), comparison *vs.* control cells (Ctr).

	Complex I OCR (Malate)					Complex II OCR (Succinate + Rotenone)				
	State 3	State 4	+ Oligo	RCR	RCR_{Oligo}	State 3	State 4	+ Oligo	RCR	RCR_{Oligo}
	(nmol O × /min × mg/protein)					(nmol O × /min × mg/protein)				
Ctr	8.11 ± 0.70	4.21 ± 0.35	0.89 ± 0.05	1.92 ± 0.09	9.19 ± 0.78	3.96 ± 0.25	3.55 ± 0.21	1.58 ± 0.11	1.12 ± 0.09	2.51 ± 0.18
Ale	5.70 ± 0.37**	3.87 ± 0.31	1.02 ± 0.01*	1.47 ± 0.10*	5.60 ± 0.38***	3.40 ± 0.27*	2.90 ± 0.21*	1.75 ± 0.11*	1.18 ± 0.13	1.94 ± 0.13**
Zol	6.32 ± 0.51*	4.08 ± 0.34	1.02 ± 0.01*	1.55 ± 0.09*	6.21 ± 0.14***	3.52 ± 0.30*	2.98 ± 0.25*	1.91 ± 0.16*	1.18 ± 0.16	1.85 ± 0.14**

Table 1. Respiratory rates and respiratory control ratios in permeabilized control (Ctr), alendronate-treated (Ale), and zoledronate-treated (Zol) endothelial cells. State 4, nonphosphorylating respiration; State 3, phosphorylating respiration in the presence of ADP; + Oligo, nonphosphorylating respiration in the presence of oligomycin; RCR, respiratory control ratio (State 3/State 4); RCRoligo, State 3 *vs.* State 4 in the presence of oligomycin (State 3/ + Oligo). Respiratory substrates: succinate (plus 2 µM rotenone) or malate. Mean ± SD; $n = 5$. $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), comparison *vs.* control cells (Ctr).

Discussion

The influence of NBPs on the oxidative metabolism of endothelial cells has yet to be determined. Our results showed that chronic 6-day exposure of endothelial cells (EA.hy926) to either 5 µM alendronate or a five-fold lower concentration of zoledronate caused similar metabolic changes that were not statistically different between the two NBPs.

Exposure of endothelial cells to higher concentrations of NBPs (above 7.5 µM for alendronate and above 2.5 µM for zoledronate) resulted in a decrease in cell viability, indicating the cellular toxicity of these compounds (Fig. 1). NBPs such as zoledronate and alendronate negatively affected angiogenesis in HUVECs by inhibiting cell adhesion, proliferation, viability, and migration[17–21]. Thus, our study supports previous research showing that higher concentrations of NBPs can cause dysfunction of the endothelium, and this may contribute to various

side effects of these anti-osteoporosis drugs due to the importance of this tissue in vascular homeostasis. In particular, long-term use or high doses of NBPs can result in adverse reactions such as osteonecrosis of the jaw, musculoskeletal pain, and atypical fractures of long bones[1,8,14,15,21].

Impaired endothelial function is characterized by increased oxidative stress and a pro-inflammatory state. Zoledronate has been shown to impede endothelial cell function and survival by inhibiting multiple prenylation-dependent signaling pathways, including ERK1/2[18]. Importantly, ERK1/2 also serves as an anti-inflammatory signal to prevent inflammatory signaling in endothelial cells[32]. The endothelial cells treated with zoledronate in our study showed a significant decrease in the level of active phospho-ERK1/2 protein (Fig. 5b) accompanied by increases in the levels of the IL6 and ICAM1 inflammatory markers (Fig. 3). These results indicate that the activation of zoledronate-treated endothelial cells triggered local inflammation by inducing the expression of inflammatory cytokines and adhesion molecules. As in our experiments, previous studies have shown that in osteoblasts and fibroblasts zoledronate, unlike alendronate (even at a five-fold lower concentration), induces an increased level of inflammatory cytokines[34]. In rat endothelial cells, 5 μM alendroante also does not elevate ICAM1[35]. Thus, these observations indicate that zoledronate induces a stronger inflammatory response compared to alendronate, although the effect of both NBPs on ERK 1/2 phosphorylation was similar.

No studies concerning the effects of NBPs on cell coenzyme Q10 levels have been reported, although NBP therapy has been associated with impaired coenzyme Q10 status in the plasma of postmenopausal women[8]. This study is the first to demonstrate that 5 μM alendronate and 1 μM zoledronate significantly reduced cellular Q10 levels, thereby depriving endothelial cells of an important antioxidant and mitochondrial electron carrier, a result that could contribute to a loss of cell viability (Figs. 1 and 2a). The ~ 30% NBP-induced decrease in total cellular Q10 content, accompanied by elimination of the reduced $Q10H_2$ pool (Fig. 2a) that functions as an antioxidant, resulted in increases in total and mitochondrial ROS formation (Fig. 2b and c). Thus, NBPs disturbed the cellular Q10 redox homeostasis, thereby decreasing the Q10 redox state ($Q10H_2$/Q10) from 0.09 to 0. In endothelial cells treated with NBPs, the Q10 deficiency was only partially restored by other antioxidants (i.e., glutathione reductase and superoxide dismutase 1), leading to oxidative stress (Fig. 2d). Thus, NBPs were able to induce a Q10 deficiency and oxidative stress in endothelial cells by inhibiting FPPS, a key enzyme of the intracellular mevalonate pathway. Previously, significant reductions in Q10 levels resulting in oxidative stress have been observed in endothelial cells treated with the statin atorvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, another important enzyme in the mevalonate pathway[7].

Our results showed that NBPs increased mitochondrial oxidative capacity and anaerobic respiration in endothelial cells in terms of CS/COX and LDH activities and expression levels, respectively. However, although there was an increase in the respiratory capacity, a decrease in ATP levels was observed in the NBP-treated cells, indicating an impairment of energy metabolism (Fig. 4e). Despite the slight increase in the mitochondrial content (the amounts of the mitochondrial proteins COX, CS, and VDAC1) (Fig. 4a), the levels of the mitochondrial biogenesis markers PGC1α and NRF2 were unchanged in the NBP-exposed cells (Fig. 5a). In addition, these cells showed a reduced level of the mitochondrial fission markers (MFF and phospho-DRP1) but no change in the level of the mitochondrial fusion marker OPA1 (Fig. 5a). Alternating fission and fusion processes help the mitochondrial network adapt to changing cellular metabolic conditions and allow for the mixing and dissemination of mitochondrial metabolites and enzymes[36]. In our study, reduced mitochondrial fission markers in NBP-treated endothelial cells could indicate less mitochondrial clearance by mitophagy resulting from the downregulation of ERK1/2 signaling (Fig. 5b), the pathway that controls mitochondrial fission and intracellular apoptosis[36]. By inhibiting FPPS and thereby preventing prenylation and activation of small GTPases, NBPs could reduce prenylation-dependent ERK1/2 signaling and the related mitochondrial fission machinery; this may influence metabolic reprogramming. Thus, NBP-induced changes in mitochondrial turnover in endothelial cells may result from dysregulated ERK signaling leading to impaired fission despite oxidative stress that supposedly promotes fission to remove damaged part by mitophagy and maintain mitochondrial function. Therefore, when mitochondrial fission is impaired due to NBPs, the mitochondrial pool may be preserved even with Q10 deficiency. However, further studies should evaluate changes in mitochondrial turnover induced by NBPs by examining mitochondrial morphology.

An overall decrease in mitochondrial respiration was observed in NBP-treated endothelial cells when stronger reducing substrates were used (pyruvate, glutamine, and a mixture of all tested substrates) (Fig. 6b), indicating a reduction in the upper limit of oxygen consumption that was possibly related to coenzyme Q10 deficiency (Fig. 2a), despite the greater total cell respiratory capacity (expressed by COX activity, Fig. 4c). With the weaker reducing substrates (glucose and palmitate) that did not reach this limit (Fig. 6b), the increase in oxidation observed was likely related to the higher mitochondrial content in the NBP-treated cells. In addition, the limitation of the mitochondrial respiratory chain function resulting from the coenzyme Q10 deficiency was indicated by significantly reduced phosphorylating respiration during the oxidation of the complex II substrate (succinate) and especially the complex I substrate (malate) in NBP-treated permebilized endothelial cells (Table 1). The increase in proton leakage in NBP-treated intact cells (Fig. 6c) and the increased nonphosphorylating respiration in the presence of oligomycin together with the decreased respiration control ratio in permeabilized NBP-treated cells (Table 1) indicate increased mitochondrial uncoupling, which can reduce the efficiency of oxidative phosphorylation (ATP synthesis). Impairment of the oxidative phosphorylation system was also indicated by reduced ATP-related respiration with stronger reducing substrates in NBP-treated intact cells (Fig. 6d), decreased ATP levels in these cells (Fig. 4e), and significantly lower phosphorylating respiration independent of the respiratory substrate in permeabilized NBP-treated cells (Table 1). These significant changes in the energy metabolism of NBP-treated endothelial cells caused a significant reduction in oxygen consumption (Fig. 6, Table 1) that in turn led to an increase in the oxygen level in the cells, as indicated by the decrease in the level of HIF1α and a six-fold increase in the expression level of KDM6A, a direct cellular oxygen sensor. NBP-induced increases in the oxygen levels of endothelial cells can contribute to oxidative stress. To our knowledge, this is the first study

that links coenzyme Q10 deficiency to increased cellular oxygen levels. Changes in the oxidative metabolism of endothelial cells due to an inhibition of the mevalonate pathway have been reported in a study concerning the statin atorvastatin[7]. Similar to NBP-induced changes (this study), the atorvastatin-induced changes in endothelial cells included coenzyme Q10 deficiency, oxidative stress induction, and decreased mitochondrial respiration[7]. Interestingly, statins did not affect mitochondrial biogenesis in endothelial cells.

In conclusion, our results confirm previous reports that higher concentrations of NBPs may negatively affect angiogenesis by inhibiting endothelial cell viability, thereby causing various side effects associated with these anti-osteoporosis drugs. In addition, our study shows for the first time that NBPs, especially zoledronate, can modulate the energy metabolism of endothelial cells, leading to alterations in the energy state of cells, the coenzyme Q10 redox balance, mitochondrial respiratory function, and mitochondrial turnover. NBPs induced metabolic reprogramming in endothelial cells in response to the deficiency of coenzyme Q10, a cellular antioxidant and a key electron carrier in the mitochondrial respiratory chain.

Material and methods

Cell culture and cell fraction preparation

We used the stable human endothelial cell line EA.hy926 (ATCC CRL-2922, ATCC, Manassas, VA, USA) that was originally derived from the human umbilical vein. Endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 2% hypoxanthine-aminopterin-thymidine (HAT), and 1% penicillin/streptomycin at 5% CO_2 and 37 °C. Cells were cultured under controlled conditions or in the presence of one of the bisphosphonates alendronate or zoledronate dissolved in water (pH 7.0). Cells from passages 4–12 were grown in 140 mm dishes to approximately 90–100% confluence. Bisphosphonates were added on the day of passage to a final concentration of 5 µM alendronate or 1 µM zoledronate (except for the studies described in Fig. 1).

After 6 days of culture, cells from the control and NBP-treated cultures were harvested with trypsin/ethylenediaminetetraacetic acid (EDTA), washed with 10% FBS in phosphate-buffered saline (PBS), 5% FBS in PBS, and finally with PBS alone, and centrifuged at 1200 × *g* for 10 min at 4 °C. Finally, the cell pellets were resuspended in cold PBS (1 g of cells per 3 ml of PBS) and maintained on ice. The yield of harvested cells was similar in the control and NBP-treated cells, with approximately 1 g of cells per 10 dishes (when all cell types were plated at the same density).

The cells were homogenized 10 times for 5 s in PBS using a polytron (IKA T18, IKA-Werke GmbH & Co. KG, Staufen, Germany) to obtain cytosolic fractions for enzyme measurements. Unbroken cells and cell debris were removed by centrifugation of the homogenates at 1200 × *g* for 10 min at 4 °C. Homogenate supernatants were collected to measure citrate synthase (CS) and lactate dehydrogenase (LDH) activities.

Enzyme activities in cytosolic fractions

Citrate synthase (CS) activity was determined spectrophotometrically by following the formation of 5,5′-dithiobis(2-nitrobenzoic acid)-Coenzyme A (DTNB-CoA) at 412 nm using a Shimadzu UV 1620 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), as previously described[7]. The reaction mixture contained 100 µM oxaloacetate, 100 µM acetyl-CoA, 100 µM DTNB, 0.1% Triton X-100, 100 mM Tris/HCl (pH 8.0) and 100 µg protein/ml cytosolic fraction.

Lactate dehydrogenase (LDH) activity was measured spectrophotometrically at 340 nm during the oxidation of 200 µM NADH in the presence of 20 mM pyruvate, 50 mM Tris/HCl (pH 7.3) and 100 µg protein/ml cytosolic fraction[37].

Enzymatic measurements were conducted at 37 °C with constant stirring.

Cell respiration

Cell oxygen consumption was measured polarographically at 37 °C using a Clark-type oxygen electrode (Hansatech Instruments Ltd, Pentney, UK) in 0.6 ml of DMEJ containing 0.8 mM $MgSO_4$, 5.4 mM KCl, 110 mM NaCl, 1.1 mM NaH_2PO_4, 44 mM $NaHCO_3$ and 10 mM Na/Na buffer (pH 7.5) as previously described[7,38]. The measurement was performed with cells at a final concentration of 4 mg protein/ml. The respiratory substrates used were 5.5 mM glucose, 5 mM pyruvate, 4 mM glutamine, 0.3 mM palmitate, and a mixture of these. To estimate proton leakage, i.e., non-ATP-linked oxygen consumption rate (OCR), ATP synthesis in basal respiration was inhibited with oligomycin (1 µg/ml). The maximal OCR was then determined by adding up to 0.5 µM carbonyl-*p*-trifluoromethoxyphenylhydrazone cyanide (FCCP), as an uncoupler. No residual (non-mitochondrial) respiration was observed in the presence of 0.5 mM cyanide.

The maximal activity of cytochrome *c* oxidase (COX) in cell fractions was measured as the rate of oxygen consumption during oxidation with up to 2 mM N,N,N′N′-tetramethyl-*p*-phenylenediamine (TMPD) in the presence of 10 µM antimycin A and 8 mM ascorbate[39].

To assess mitochondrial respiration, we measured the oxidation of the complex I (5 mM malate) and complex II (5 mM succinate plus 2 µM rotenone) substrates in endothelial cells permeabilized with 0.02% digitonin. The measurement was performed with cells at a final concentration of 6.7 mg protein/ml in 0.6 ml of incubation medium (at 37 °C) containing 150 mM sucrose, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM Tris/HCl (pH 7.2) and 0.1% bovine serum albumin (BSA). Phosphorylating (state 3) respiration was measured in the presence of 150 µM ADP. Nonphosphorylating (resting state, state 4) respiration was measured in the absence or presence of oligomycin (1 µg/mg/protein) an inhibitor of ATP synthase. Respiratory control ratio (RCR) was calculated as the state 3 respiratory rate attained during maximal ATP synthesis (i.e., in the presence of ADP) divided by the respiratory rate in the absence of added ADP (in the presence or absence of oligomycin).

Cell viability
A 0.4% trypan blue solution (1:1 v/v) was added to the harvested live and dead endothelial cells and cell viability was determined using a Countess Automatic Cell Counter (Invitrogen, Carlsbad, CA, USA).

ATP level detection
ATP levels in endothelial cells were measured using the Luminescent ATP Detection Assay kit (ab113849, Abcam, Cambridge, UK). After cell lysis, luciferase and luciferin were added and the emitted light was detected using a Tecan Spark multiplate reader (Tecan Group Ltd, Mannedorf, Switzerland).

ROS formation
Total cellular ROS formation was measured with a 5 mM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) probe, and mitochondrial ROS (superoxide) formation was determined using a 5 µM MitoSox Red probe. Cells (50 µg protein/ml) were incubated with fluorescent probes in PBS containing 5.5 mM glucose and 5 mM pyruvate for 10 min at 37 °C. The cells were then washed twice with PBS, centrifuged (1200 × *g* for 10 min at 4 °C) and resuspended in PBS to a concentration of 50 µg protein/ml. Measurements were performed in 96-well plates with MitoSox (Excitation/Emission at 510/595 nm) and CM-H2DCFDA (Excitation/Emission at 495/522 nm) using a Tecan Spark multiplate reader (Tecan Group Ltd).

Cellular Q10 concentration
Cellular Q10 levels were determined by extraction and high-performance liquid chromatography (HPLC), as previously described[40,41] using a LiChrosorb RP-18 (10 µm) HPLC column (Hichrom, Theale, UK). Both the reduced (275 nm) and oxidized (290 nm) forms of Q10 were detected. Commercial Q10 was used to quantify and calibrate the Q10 peaks. Total and reduced Q10 pools were determined in endothelial cells under fully oxidizing conditions, i.e., in the absence of Q-reducing respiratory substrates. Prior to Q extraction, the cells (30 mg) were incubated with gentle agitation for 10 min in 3 ml of PBS.

Protein immunodetection
Endothelial cells were lysed with RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS and 50 mM Tris/HCl, pH 8.0). 8–12% SDS-PAGE gels were used for protein separation. The Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular weight marker. The following primary antibodies were obtained from Abcam: rabbit polyclonal anti-citrate synthase (CS, 46 kDa) (ab96600), anti-cytochrome *c* subunit II (COXII, 24 kDa) (ab110258), anti-voltage-dependent anion-selective channel protein 1 (VDAC1, 35 kDa) (ab14734), anti-nuclear factor erythroid 2-related factor (NRF2, 68 kDa) (ab137550), anti-peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α, 92 kDa) (ab54481), anti-mitochondrial cleavage factor (MFF, 37 kDa) (ab81127), anti-glutathione reductase (GR, 50 kDa) (ab128933), anti-superoxidase dismutase 1 (SOD1, 18 kDa) (ab13498), anti-intercellular adhesion molecule 1 (ICAM1, 90 kDa) (ab53013), anti-mitochondrial marker (mitochondrial non-glycosylated protein (MTC02, 60 kDa) (ab3298), and anti-interleukin-6 (IL6 50 kDa) (ab9324). In addition, we used primary antibodies from Thermo Fisher Scientific: anti-lysine (K)-specific demethylase 6A (KDM6A, 140 kDa) (PA5-68598), anti-hypoxia-inducible factor 1-alpha (HIF1α, 115 kDa) (PA5-85494), anti-artrophy-1 protein (OPA1, 100 and 80 kDa) (BDB612607), anti-phospho-dynamin related protein 1 (Ser616) (Phospho-DRP1) (PA5-64821, 95 kDa), and anti-lactate dehydrogenase (LDH, 35 kDa) (PA5-27406). Cell Signaling Technology (Danvers, MA, USA) provided antibodies: anti-extracellular signal-regulated protein kinase (ERK1/2, 42/44 kDa) (#4695), anti-phospho-ERK1/2 (Thr202/Tyr204), (42/44 kDa) (#9101), Merck (Darmstadt, Germany) provided anti- dynamin related protein 1 (DRP1, 74–95 kDa) (ABT155), while the anti-hexokinase I (HK I, 120 kDa) (sc-80978) was obtained from Santa Cruz Biotechnology (Dallas, TX, US). The appropriate horseradish peroxidase-conjugated secondary antibodies were used. The expression levels of anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 37 kDa) (ab9485) and β actin (42 kDa) (CP01, Calbiochem), as well as Ponceau staining were used as loading controls for normalization. The blots were cut before hybridization with antibodies during blotting. Uncropped images of blots and exemplary blot images used for densitometric analysis are shown in Supplementary Figs. S2, S3, S4, 5a and 5b. Protein bands were visualized using the SuperSignal ECL substrate (Thermo Fisher Scientific) and were digitally quantified using the ImageJ software package.

Statistical analysis
Data are presented as means ± SD of 5–10 independent preparations of cell suspensions. Each measurement was performed in at least two replicates. ANOVA (followed by Tukey's post hoc comparisons for $P < 0.05$) or nonparametric Kruscal-Wallis ANOVA (followed by Dunn's post hoc comparisons for $P < 0.05$) was used to determine statistically significant differences ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Data availability
All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

Conceptualization, A.B., L.G., W.J..; investigation, A.B. (all methods), L.G. (protein immunoblotting); formal analysis, A.B., L.G., W.J.; writing and visualization, W.J., A.B., L.G.; critical discussion, A.B., L.G., W.J.; supervision and funding acquisition, W.J. All authors have read and agreed to the published version of the manuscript.

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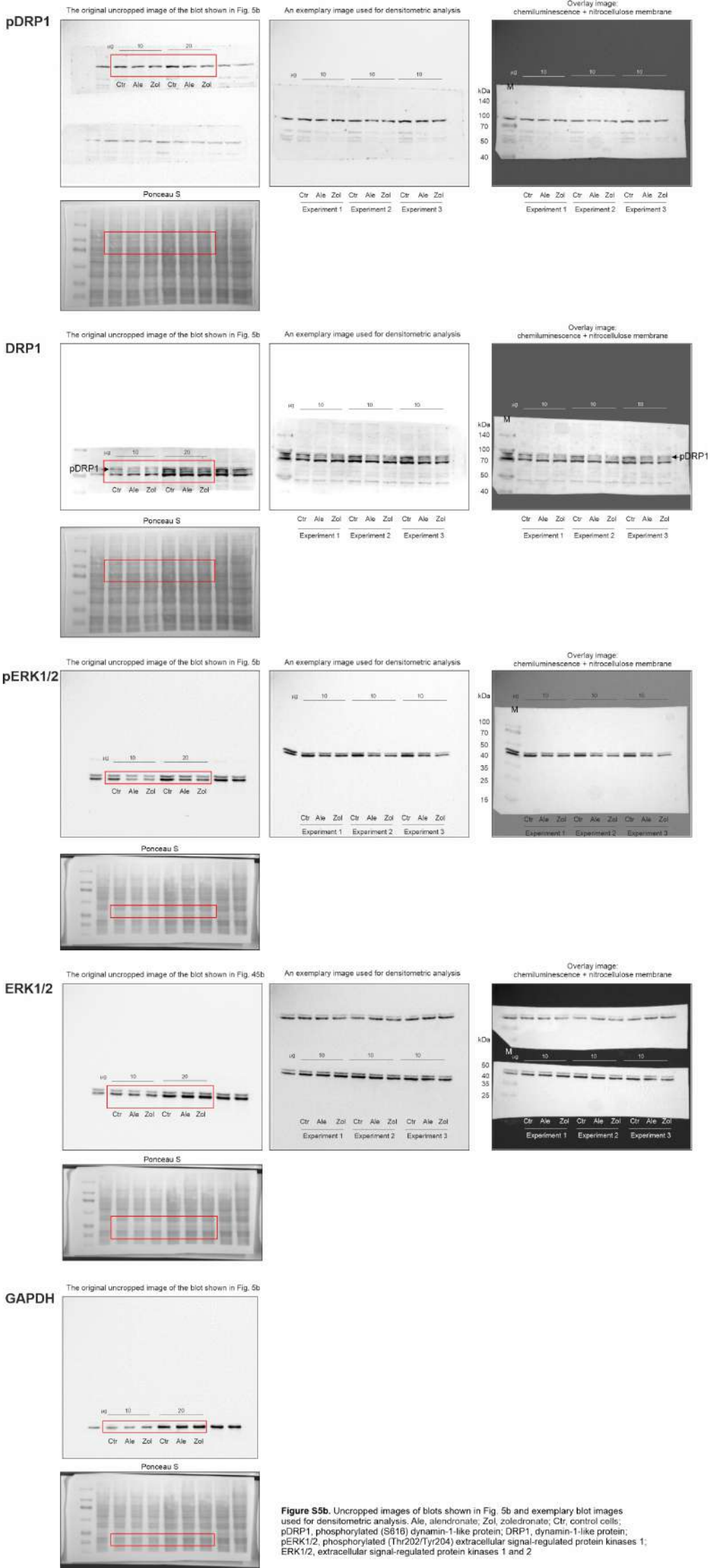


Figure S5b. Uncropped images of blots shown in Fig. 5b and exemplary blot images used for densitometric analysis. Ale, alendronate; Zol, zoledronate; Ctr, control cells; pDRP1, phosphorylated (S616) dynamin-1-like protein; DRP1, dynamin-1-like protein; pERK1/2, phosphorylated (Thr202/Tyr204) extracellular signal-regulated protein kinases 1; ERK1/2, extracellular signal-regulated protein kinases 1 and 2

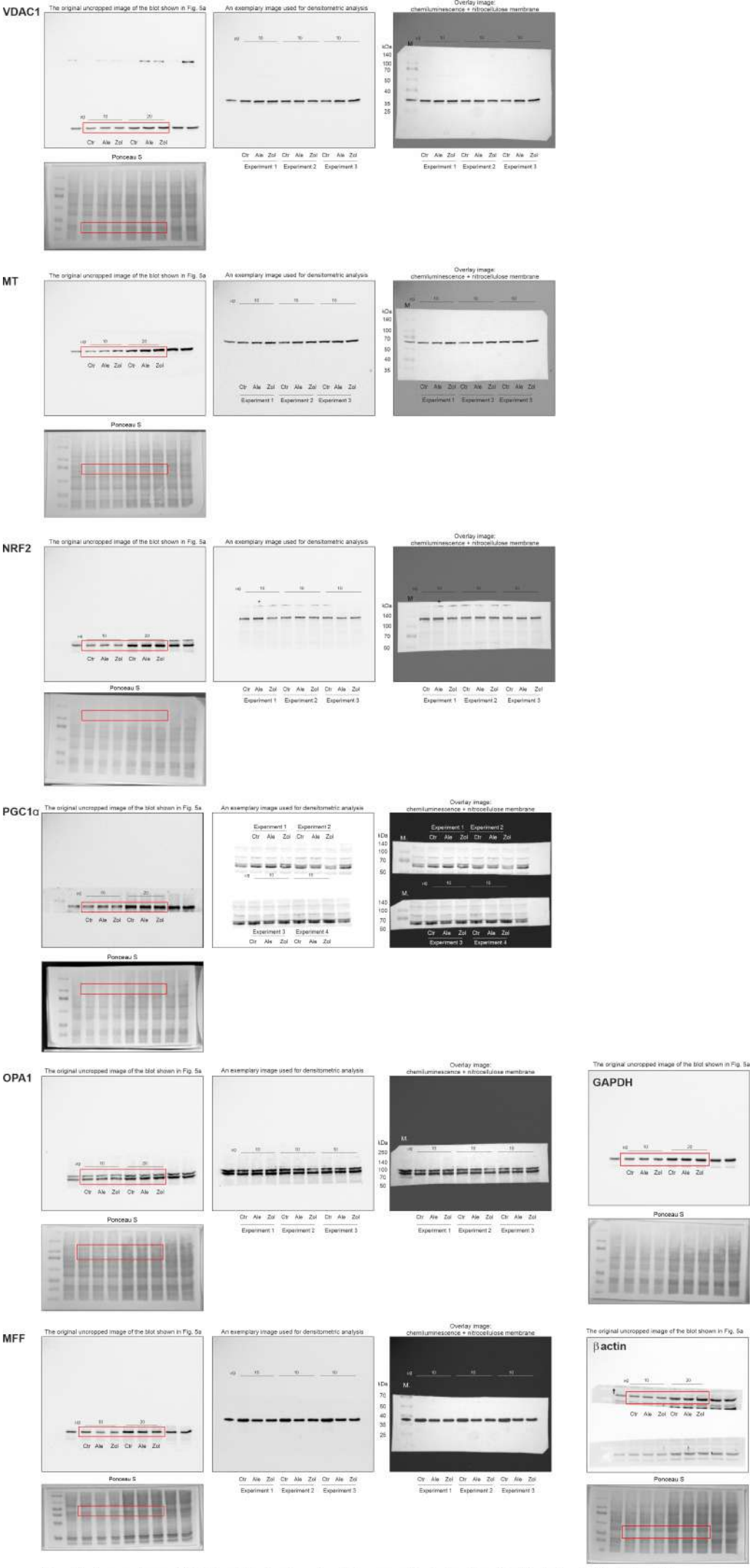


Figure S5a. Uncropped images of blots shown in Fig. 5a and exemplary blot images used for densitometric analysis. Ale, alendronate; Zol, zoledronate; Ctr, control cells; VDAC1, voltage-dependent anion-selective channel protein 1; MT, mitomarker; NRF2, nuclear factor erythroid 2-related factor, PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; OPA1, artrophy-1 protein; MFF, mitochondrial cleavage factor.

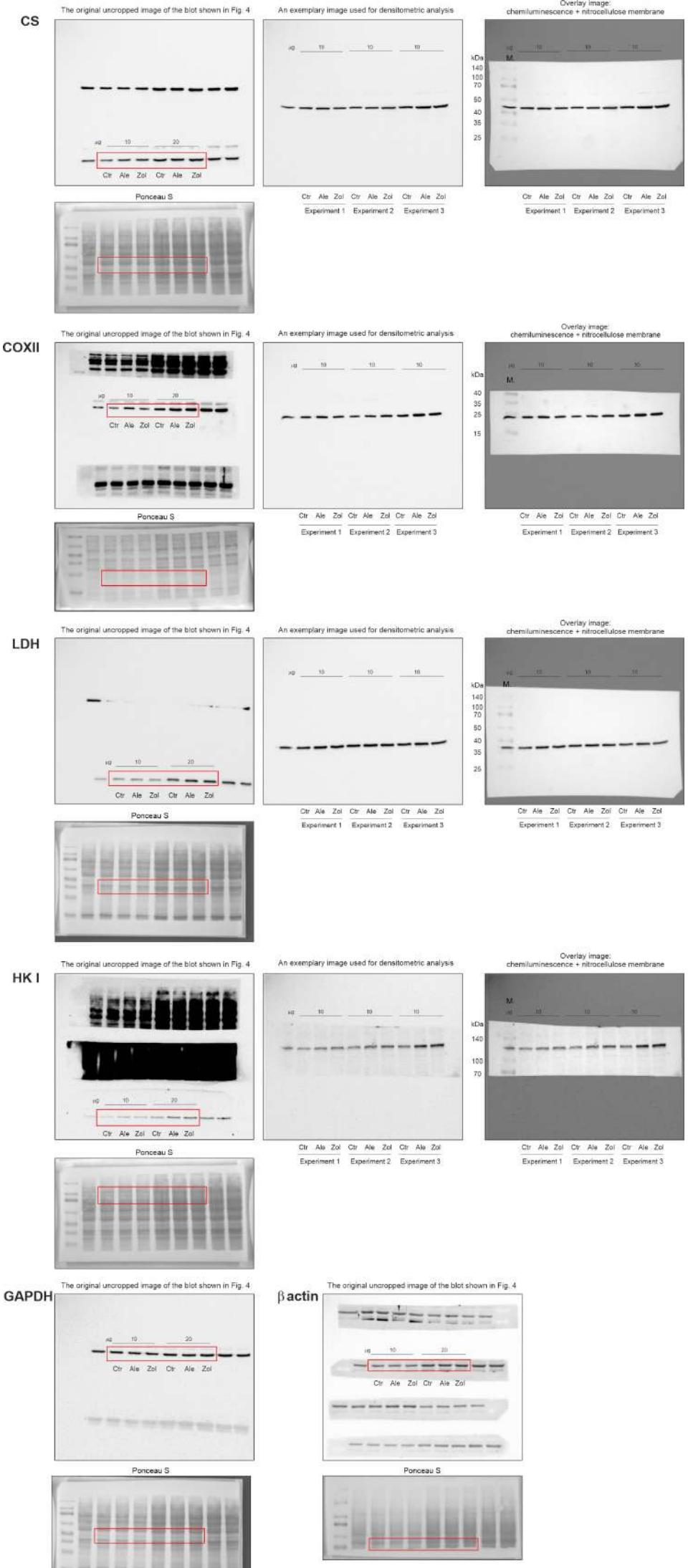
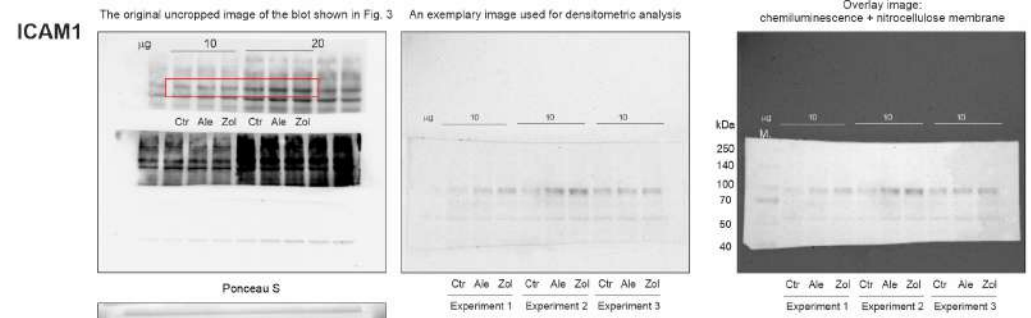
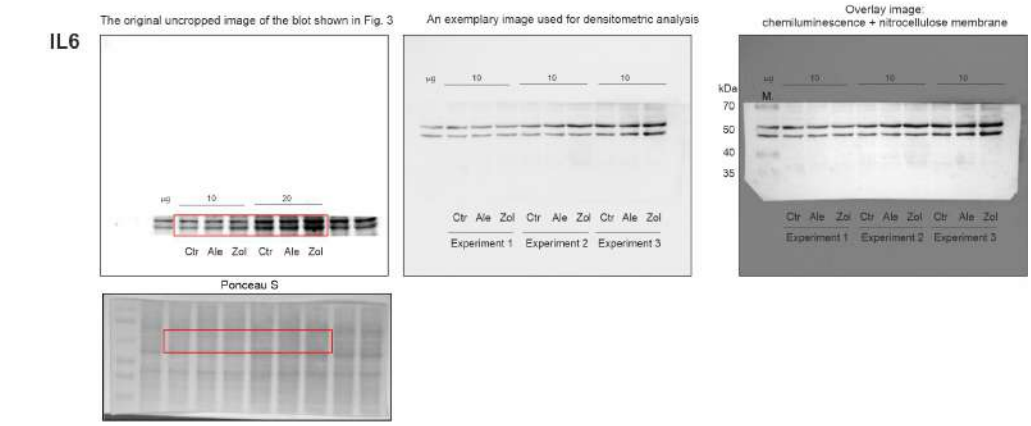
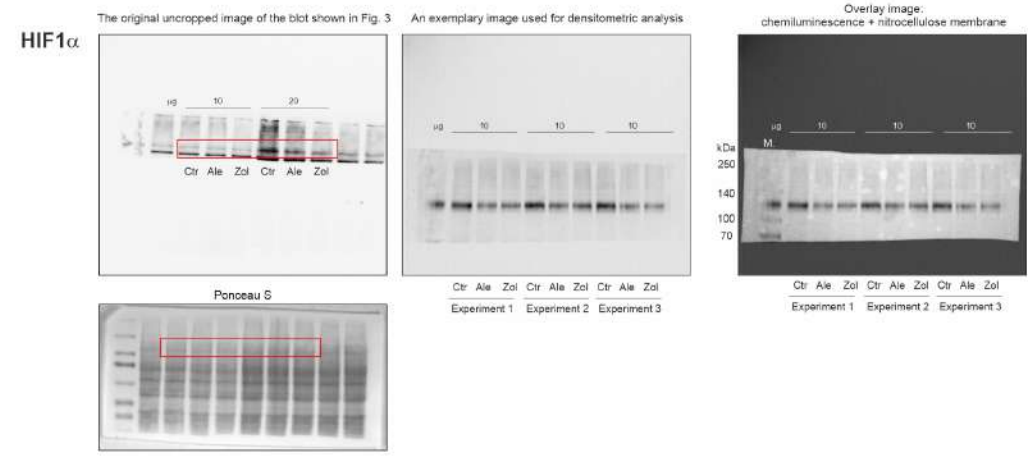
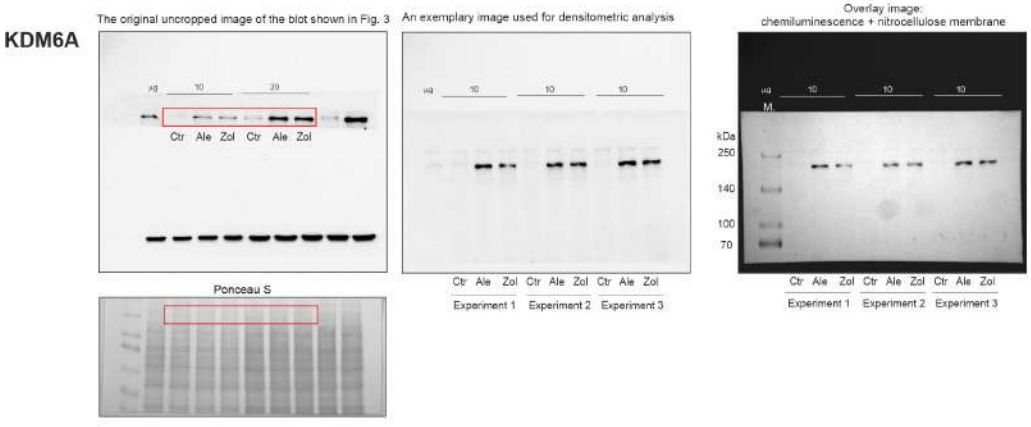


Figure S4. Uncropped images of blots shown in Fig. 4a and exemplary blot images used for densitometric analysis. Ale, alendronate; Zol, zoledronate; Ctr, control cells; CS, citrate synthase; COXII, cytochrome c subunit II; LDH, lactate dehydrogenase; HK I, hexokinase I.









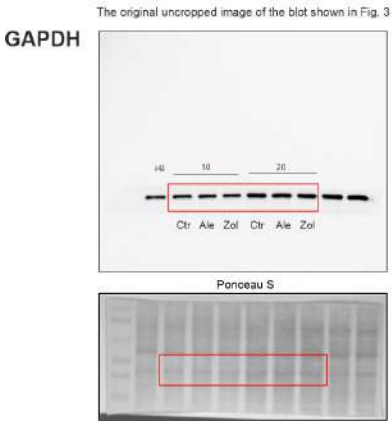
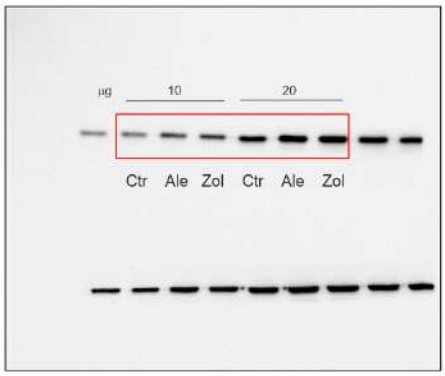


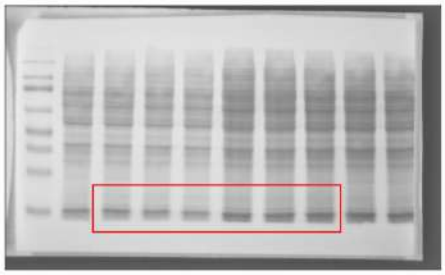
Figure S3. Uncropped images of blots shown in Fig. 3 and exemplary blot images used for densitometric analysis. Ale, alendronate; Zol, zoledronate; Ctr, control cells; ICAM1, intercellular adhesion molecule 1; IL6, interleukin-6; HIF1α, hypoxia-inducible factor 1α; KDM6A, lysine (K)-specific demethylase 6A.

SOD1

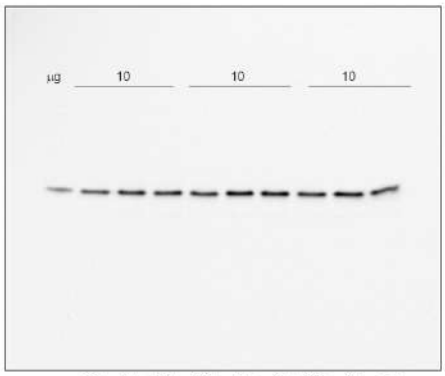
The original uncropped image of the blot shown in Fig. 2d



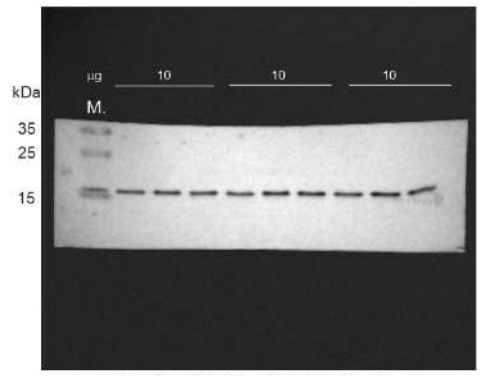
Ponceau S



An exemplary image used for densitometric analysis

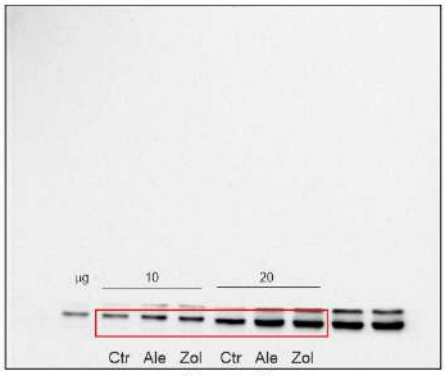


Overlay image:
chemiluminescence + nitrocellulose membrane

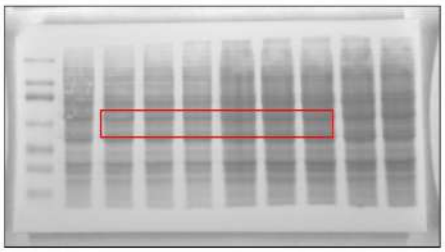


GR

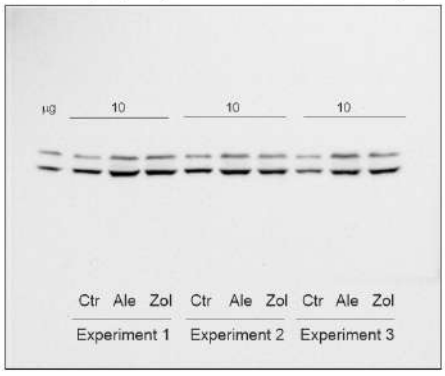
The original uncropped image of the blot shown in Fig. 2d



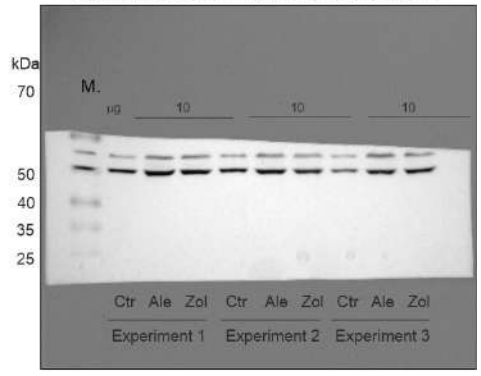
Ponceau S



An exemplary image used for densitometric analysis

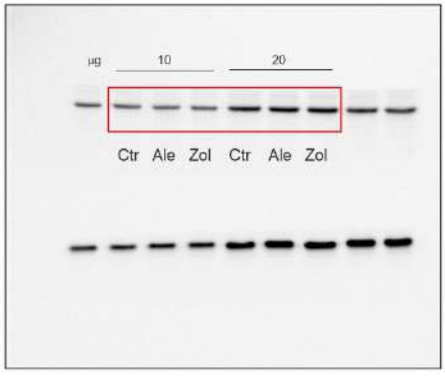


Overlay image:
chemiluminescence + nitrocellulose membrane



GAPDH

The original uncropped image of the blot shown in Fig. 2d



Ponceau S

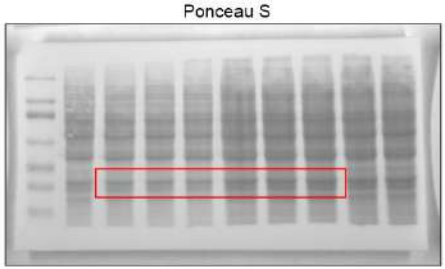


Figure S2 Uncropped images of blots shown in Fig. 2d and exemplary blot images used for densitometric analysis. Ale, alendronate; Zol, zoledronate; Ctr, control cells; SOD1, superoxide dismutase; GR, glutathione reductase.

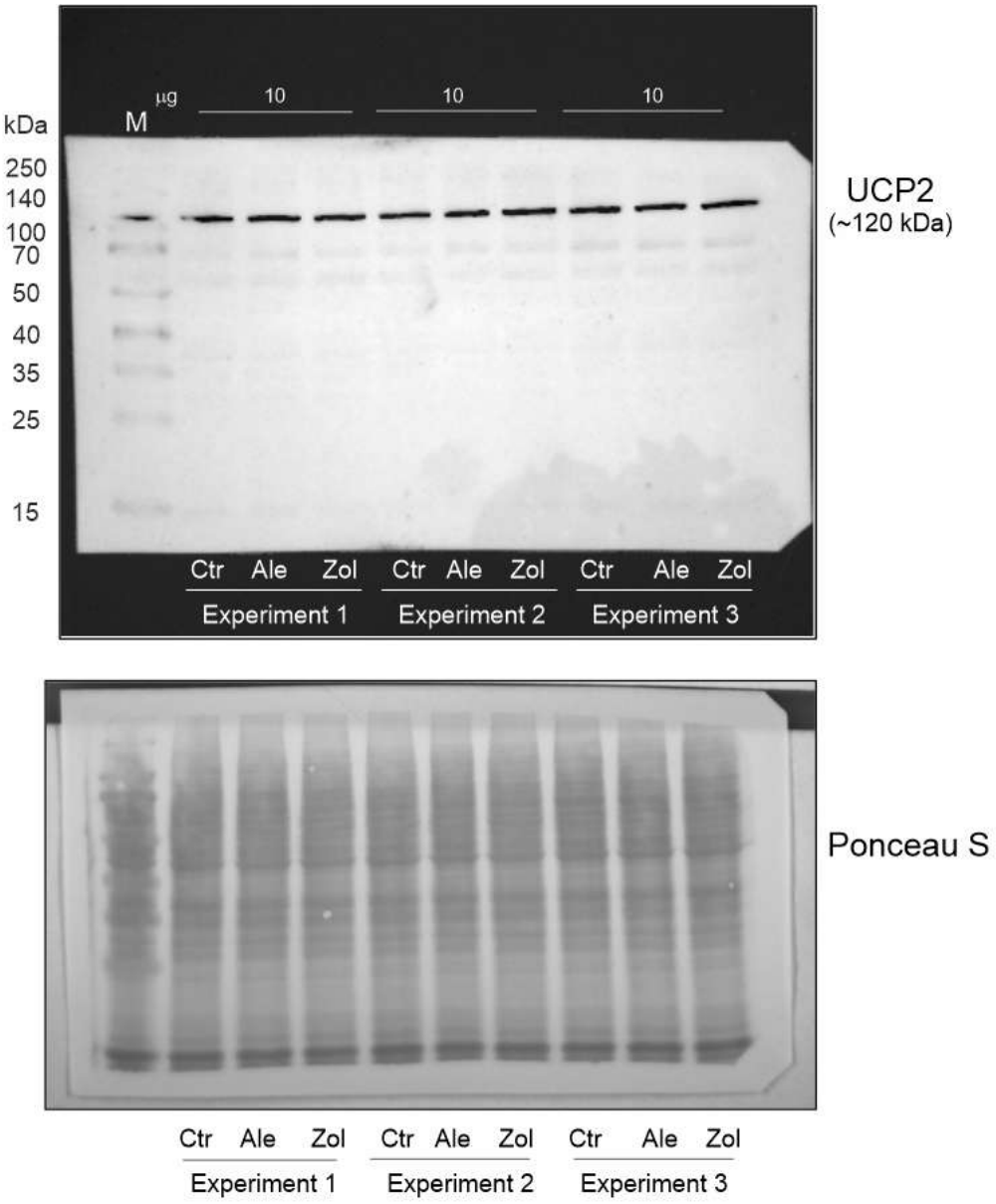


Figure S1 A 6-day endothelial cell culture with 5 µM alendronate (Ale) and 1 µM zoledronate (Zol) did not change level of uncoupling protein 2 (UCP2). Representative uncropped blot and Ponceau S staining are presented. UCP2 tetramer (~120 kDa) was detected. 10 µg of cell lysate protein was loaded per lane. Ctr, control cells. Anti-UCP2 antibody (Abcam, ab97931) was used.

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Adaptation of mitochondrial bioenergetics to coenzyme Q deficiency in human endothelial cells after chronic exposure to bisphosphonates

Adrianna Budzinska, Lukasz Galganski✉, Krzysztof Wojcicki & Wieslawa Jarmuszkiewicz✉

Nitrogen-containing bisphosphonates (N-BPs), widely used in bone disease therapy, inhibit the mevalonate pathway, which affects coenzyme Q (CoQ) biosynthesis and may compromise mitochondrial function, particularly in endothelial cells where oxidative stress and mitochondrial dysfunction contribute to cardiovascular disease. This study examined the effects of chronic six-day exposure of human endothelial cells to N-BPs on mitochondrial bioenergetic functions, focusing on drug-induced mitochondrial CoQ (mtCoQ) deficiency. Compared with the mitochondria of control cells, those of endothelial cells treated with 5 µM alendronate or 1 µM zoledronate presented a significant 45–50% decrease in total mtCoQ pool, loss of reduced ($mtCoQH_2$) antioxidant mtCoQ pool, and elevated mitochondrial antioxidant protein superoxide dismutase 2 (SOD2) and uncoupling protein 2 (UCP2) levels. Exposing endothelial cells to N-BPs also led to an overall reduction in mitochondrial substrate oxidation, except for increased fatty acid oxidation. Additionally, the mitochondria of N-BP-treated endothelial cells presented decreased respiratory rates, membrane potential, and ATP synthesis efficiency, and increased H_2O_2 production resulting from increased mtCoQ reduction during the oxidation of complex I (CI) and CII substrates. N-BP-induced mtCoQ deficiency also resulted in rearranged respiratory chain supercomplexes, particularly downregulation of the III_2 + IV supercomplex, and decreased CII, CIII, and CV protein levels and activities. Despite the N-BP-induced decrease in *a*-heme levels, maximal CIV activity remained unaffected in endothelial mitochondria. These findings highlight the role of N-BPs in disrupting mtCoQ redox homeostasis and associated bioenergetic functions in endothelial mitochondria.

Keywords Alendronate, Bisphosphonates, Coenzyme Q, Endothelial cells, Mitochondrial respiration, Zoledronate

Abbreviations

ACADS	Acyl-coenzyme A dehydrogenase
CoQ	Coenzyme Q
CoQ10	Coenzyme Q10
CoQ10B	Coenzyme Q-binding protein CoQ10 homolog B
CS	Citrate synthase
COX	Cytochrome *c* oxidase, complex IV
CI-CIV	Complexes of respiratory chain
FPPS	Farnesyl diphosphate synthase
GDH	Glutamate dehydrogenase
$mitoBK_{Ca}$	Mitochondrial large-conductance Ca^{2+}-activated potassium channel
mtCoQ	Mitochondrial CoQ
$mtCoQH_2$/mtCoQtot	MtCoQ reduction level (reduced mtCoQ/total mtCoQ)

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N-BP(s)	Nitrogen-containing bisphosphonates
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species
mtROS	Mitochondrial ROS
SOD2	Superoxide dismutase 2
UCP(2, 3)	Uncoupling protein (2,3)
ΔΨ	Mitochondrial transmembrane electrical potential

Mitochondria play a key role in cellular bioenergetics, containing the respiratory chain responsible for ATP production through oxidative phosphorylation (OXPHOS), the final step in aerobic respiration. Coenzyme Q (CoQ) is a critical electron carrier in the mitochondrial respiratory chain and an important antioxidant found in all cell membranes[1]. Nevertheless, mitochondrial CoQ (mtCoQ) also generates mitochondrial reactive oxygen species (mtROS) within the respiratory chain. mtCoQ is directly involved in the formation of superoxide/hydrogen peroxide at four mtCoQ-binding sites, i.e., the mtCoQ-reducing site of complex I (I_Q) (both during forward and reverse electron transfer)[2,3], mitochondrial glycerol-3-phosphate dehydrogenase (G_Q)[4], dihydroorotate dehydrogenase (D_Q)[5], and the mtQH$_2$-oxidizing site of complex III (III_{Qo})[6]. Mitochondrial dysfunction, including that associated with decreased CoQ levels, has been linked to cellular dysfunction and several metabolic, cardiovascular, and neurodegenerative diseases[7,8].

Bisphosphonates, synthetic analogs of pyrophosphate, are frequently used to manage osteoporosis and other bone disorders because they suppress bone resorption mediated by osteoclasts[9,10]. Bisphosphonates are divided into non-nitrogen-containing and nitrogen-containing bisphosphonates (N-BPs)[11]. N-BPs can be further divided into second-generation (including alendronate) and more efficient third-generation (including zoledronate) drugs. N-BPs target and inhibit farnesyl diphosphate synthase (FPPS), a critical enzyme in the intracellular mevalonate pathway, thereby interfering with the prenylation/activation of small GTPases necessary for osteoclast survival and functionality[9–11]. The mevalonate pathway is a crucial metabolic route that provides isoprenoid units and plays a central role in the biosynthesis of important molecules, including cholesterol, steroid hormones, heme *a*, and CoQ[12,13]. Decreased mtCoQ levels due to inhibition of the mevalonate pathway may lead to dysfunctional mitochondrial respiration and increased oxidative stress, as has been described for statins, which are cholesterol-lowering drugs[1,14–17]. Statins inhibit the first step of the mevalonate pathway catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and reduce the biosynthesis of isoprenoid intermediates, including those required for CoQ synthesis, leading to mtCoQ deficiency and impaired electron transport chain function, which has been described mainly in myocytes and endothelial cells[1,14–17]. N-BPs also inhibit the mevalonate pathway that supplies the isoprenoid unit necessary for CoQ synthesis; however, limited research has been conducted on the effect of N-BPs on cellular CoQ levels. N-BP therapy is associated with decreased plasma CoQ levels in postmenopausal women[18]. Furthermore, we recently demonstrated that the N-BPs alendronate and zoledronate significantly reduce total CoQ levels (i.e., CoQ from all membranes) in cultured human endothelial cells[19].

Endothelial cells lining the blood vessel interior come into contact with circulating drugs, such as N-BPs. Alterations in blood serum composition can influence the oxidative metabolism of endothelial cells, potentially resulting in oxidative stress. Endothelial dysfunction is closely related to the overproduction of reactive oxygen species (ROS), especially mitochondrial ROS (mtROS), which may promote the development of cardiovascular diseases[20–23]. While N-BPs act primarily on the skeletal system, research indicates that these FPPS inhibitors may exert unintended effects on nonskeletal tissues and affect the endothelial cell functions crucial for maintaining vascular homeostasis and metabolism[24,25]. Long-term use of N-BPs or high doses may lead to adverse effects, including an increased risk of cardiovascular events or bisphosphonate-related osteonecrosis of the jaw due to the inhibition of angiogenesis[26,27].

N-BPs, especially zoledronate, have been reported to affect endothelial cell migration, survival and apoptosis[28]. Moreover, in endothelial cells, N-BPs impede angiogenesis by disrupting protein prenylation, affecting endothelial cell adhesion, proliferation, survival, migration, and actin stress fibers[29–32]. We recently reported that alendronate and zoledronate influence cellular energy and redox status in endothelial cells by significantly decreasing cellular CoQ levels, alternating cellular respiration and mitochondrial turnover, reducing cellular ATP levels, and increasing ROS production[19]. Although we previously reported that N-BPs cause a deficiency in total CoQ in endothelial cells[19], the present study is, to our knowledge, the first to investigate mtCoQ deficiency and its downstream bioenergetic consequences, including the redox state of mtCoQ, mtROS formation, and oxidative phosphorylation (OXPHOS) system remodeling. Understanding how N-BPs influence endothelial mitochondrial bioenergetic function is important for elucidating their potential effects on the cardiovascular system.

This study investigated the effects of chronic exposure to alendronate and zoledronate on mitochondrial bioenergetic functions in cultured human endothelial cells, focusing on N-BP-induced mtCoQ deficiency. We measured mitochondrial respiratory activities, membrane potential (ΔΨ), OXPHOS efficiency, uncoupling, mtROS production, mtCoQ content and reduction levels, and the molecular organization of OXPHOS components to elucidate whether prolonged treatment with N-BPs induces mitochondrial adaptations and how they may be linked to mtCoQ availability.

Materials and methods

Cell culture

Experiments were conducted using the EA.hy926 human endothelial cell line (ATCC CRL-2922, RRID: CVCL_3901, ATCC, Manassas, VA, USA) derived from the human umbilical vein. The cells were cultured for

six days under standard conditions or exposed to N-BPs, specifically 5 μM alendronate or 1 μM zoledronate, following previously established protocols[19].

Mitochondria isolation

Mitochondria were isolated as previously described[15,33] with minor modifications. After six days of culture, the cells from the control and N-BP-treated groups were harvested using trypsin/ethylenediaminetetraacetic acid (EDTA). The cells were then washed with 10% FBS (in phosphate-buffered saline, [PBS]), 5% FBS, and PBS alone. The samples were subsequently centrifuged at 1,200 × *g* for 10 min at 4 °C. The resulting cell pellets were resuspended in medium (PREPI) comprising 0.25 M sucrose, 1.5 mM EDTA, 1.5 mM ethylene glycol bis(2-aminoethyl)tetraacetic acid, 0.2% BSA, and 5 mM Tris/HCl (pH 7.2). The cells were homogenized via 17 passes with a Dounce homogenizer. After the homogenates were centrifuged at 1,200 × *g* for 10 min, the pellet was resuspended, subjected to additional homogenization (12 passes), and centrifuged again to extract the remaining mitochondria. The supernatants with crude mitochondria were then centrifuged at 12,000 × *g* for 10 min. The mitochondrial pellets were washed with PREPII medium (0.25 M sucrose and 15 mM Tris/HCl [pH 7.2]) and centrifuged again at 12,000 × *g* for 10 min. The final mitochondrial pellets were resuspended in PREPII medium.

ΔΨ and mitochondrial respiration

ΔΨ and mitochondrial respiration in isolated endothelial mitochondria were assessed as previously described[15,33]. ΔΨ was recorded simultaneously with oxygen consumption via a tetraphenylphosphonium (TPP)-specific electrode. Oxygen uptake was determined polarographically via a Rank Bros. (Cambridge, UK) oxygen electrode or a Hansatech (King's Lynn, UK) oxygen electrode. Measurements were conducted in 0.6 or 3.0 mL of a standard incubation medium (20 mM Tris/HCl, pH 7.2, 150 mM sucrose, 4 mM $MgCl_2$, 2.5 mM KH_2PO_4, and 0.1% BSA) with either 0.5 or 2.5 mg of mitochondrial protein, respectively, at 37 °C.

Phosphorylating (state 3) respiration was assessed with either 150 μM ADP (ADP/O measurements) or 1.7 mM ADP (maximal phosphorylating respiration), whereas uncoupled respiration was assessed with up to 0.9 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). Non-phosphorylating (state 4, resting state) respiration was measured without ADP. The respiratory substrates used were 4 mM malate (complex I, CI, substrate), 4 mM succinate plus 1.2 μM rotenone (CII substrate), 4 mM succinate plus 4 mM malate, 0.4 mM duroquinol plus 1.2 μM rotenone (CIII activity measurements), 4 mM glutamate, and 20 μM palmitoylcarnitine plus 2 mM carnitine.

The maximal activity of complex IV (CIV, cytochrome *c* oxidase [COX]) was assessed by measuring the oxygen consumption rate using 0.2 mg of mitochondrial protein (0.33 mg/mL). The assay involved the sequential addition of 5 μM antimycin A, 5 mM ascorbate, 0.04% cytochrome *c*, and up to 1 μM *N*,*N*,*N*',*N*'-tetramethyl-*p*-phenylenediamine.

UCP and mitoBK$_{Ca}$ activity

The activity of the uncoupling protein (UCP) or high-conductance Ca^{2+}-activated mitochondrial potassium channel (mitoBK$_{ca.}$) in response to the driving force was quantified by measuring the oxygen consumption rate's dependence on ΔΨ during respiratory chain inhibition titration[33]. The respiratory rate was progressively decreased by inhibiting malate and succinate oxidation with increasing concentrations of rotenone (a complex I inhibitor) and malonate (a complex II inhibitor). The inhibitors were introduced in two stages: first, 0.3 μM rotenone combined with 1 mM malonate, followed by 0.6 μM rotenone paired with 2 mM malonate. Non-phosphorylating respiration was measured in the presence of ATP synthase and ATP/ADP translocase inhibitors (1 μg/mL oligomycin and 1 μM carboxyatractyloside, respectively) to eliminate ATP turnover-dependent proton leakage. For UCP activity measurements, carboxyatractyloside also prevented inducible fatty acid-mediated proton leakage through ATP/ADP translocase. UCP activity was determined by using 8 μM linoleic acid as an activator and 2 mM GTP as an inhibitor. MitoBK$_{ca}$ activity was assessed by using 1 μM NS11021 as an activator and 2 μM iberiotoxin (IbTx) as an inhibitor. The linoleic acid-induced GTP-inhibited UCP activity and NS11021-induced IbTx-inhibited mitoBK$_{Ca}$ activity were determined at the highest common ΔΨ value from the flux-force kinetic relationship.

Mitochondrial CoQ content and reduction level

The mtCoQ content and reduction level were measured via extraction method followed by high-performance liquid chromatography analysis with a LiChrosorb RP-18 (10 μm) column and a GE Acta Explorer system (GE Healthcare, Chicago, IL, USA)[34]. Both oxidized (290 nm) and reduced (275 nm) forms of coenzyme Q10 (CoQ10) were detected. Commercial CoQ10 was used for peak calibration. The reduction level of mtCoQ ($mtCoQH_2$/mtCoQtot), which indicates the proportion of reduced mtCoQ to total mtCoQ in isolated endothelial mitochondria during steady-state respiration, was calculated. Samples for mtCoQ extraction were collected while monitoring oxygen consumption and changes in ΔΨ.

Mitochondrial H_2O_2 production

Mitochondrial H_2O_2 production was measured using the Amplex Red assay (Invitrogen, Waltham, MA, USA) in a 24-well plate, read with a Tecan multimode reader (Tecan Group Ltd., Männedorf, Switzerland). The assay was conducted at 37 °C in 0.5 mL of a standard incubation medium containing 0.1 U/mL horseradish peroxidase, 5 μM Amplex Red, and 1 U/mL superoxide dismutase (SOD) with 0.5 mg mitochondrial protein (1 mg/mL). Fluorescence was recorded over 35 min at 545 nm (excitation) and 590 nm (emission). The mitochondria were exposed to respiratory substrates (4 mM malate, 4 mM succinate plus 1.2 μM rotenone, and 4 mM malate

plus 4 mM succinate) with or without 1.5 mM ADP (phosphorylating and non-phosphorylating conditions, respectively). Known H_2O_2 concentrations were used for calibration to determine the rate of H_2O_2 production.

Cytochrome $a + a_3$ reduction

Endothelial mitochondria were resuspended at a final concentration of 5 mg protein/mL in a buffer (120 mM KCl, 10 mM Tris/HCl [pH 7.2], and 2.6 mM cyanide) and transferred to either the experimental or reference cuvette. Dithionite (0.15%) was added to the experimental cuvette (complete reduction), and $K_3[Fe(CN)_6]$ (1 mM) was added to the reference cuvette (complete oxidation). The reduced minus oxidized difference spectra of cytochromes $a + a_3$ were determined using a Shimadzu 1620 UV spectrophotometer (580–605 nm).

Mitochondrial protein level immunodetection

Proteins from isolated mitochondria were separated using 8–11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). Abcam (Cambridge, UK) primary antibodies were used to immunodetect the following proteins: cytochrome *c* oxidase subunit II (COXII; 20 kDa; ab110258), citrate synthase (CS; 46 kDa) (ab96600)uncoupling protein 2 (UCP2; (33 kDa; ab97931), uncoupling protein 3 (UCP3; 34 kDa; ab3477), coenzyme Q-binding protein CoQ10 homolog B (CoQ10B; 27 kDa; ab41997), acyl-coenzyme A dehydrogenase (ACADS; 41 kDa; ab156571), glutamate dehydrogenase (GDH; 61 kDa; ab89967), and total OXPHOS human WB antibody cocktail (ab110411), which contains antibodies against subunits of CI (18 kDa subunit NADH: ubiquinone oxidoreductase subunit B8 [NDUFB8]), CII (36 kDa subunit succinate dehydrogenase complex iron sulfur subunit B [SDHB]), CIII (subunit Core 2, 42 kDa), CIV (COXII; 20 kDa), and ATP synthase (complex V [CV] subunit α, 52 kDa). The anti-superoxide dismutase 2 (SOD2; 25 kDa) antibody (ADI-SOD) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Alomone Labs (Jerusalem, Israel) antibodies raised against the mitoBK$_{Ca}$ subunits KCa1.1 (105 kDa; APC-107) and sloβ2 (42 kDa; APC-034) were used. The protein levels of COXII or CS were used as loading controls. Images of immunodetection results shown in the figures have been cropped to standardize the presentation, since the major loading control protein (COXII, 20 kDa) has a molecular mass similar to that of most of the proteins tested. The absence of images of adequate length in the figures is also due to the fact that after protein transfer the membranes were cut to allow separate immunodetection of target proteins and loading controls, due to the limited amount of mitochondrial material available. The original immunoblots can be found in Supplementary Information (Figs. S1–S5). Protein levels were digitally quantified using ImageJ software (US National Institutes of Health, Bethesda, MD, USA).

BN–PAGE and in-gel activity assays

In-gel activity assays of complexes CI, CII, CIV, and CV following blue native polyacrylamide gel electrophoresis (BN–PAGE) separation (3–9.5% gradient gels) of mitochondrial proteins (100 µg) were conducted as previously described[15]. In addition, the BN-PAGE-separated proteins were transferred onto nitrocellulose membranes to determine the OXPHOS complexes by immunoblotting with an anti-UQCRC2 antibody (against CIII; ab14745, Abcam) or the Total OXPHOS Human WB Antibody Cocktail.

Statistical analysis

The data are expressed as the means ± standard deviations (SD), and are based on a minimum of 4–8 independent mitochondrial isolations. Each measurement was conducted at least in triplicate. Significance was evaluated via ANOVA (followed by Tukey's post hoc comparisons for $P < 0.05$) or nonparametric Kruscal–Wallis ANOVA (KW ANOVA) (followed by Dunn's post hoc comparisons for $P < 0.05$) to determine statistically significant differences ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Results

For this study, we chose two representative N-BPs: zoledronate, recognized for its high potency attributed to its unique R2 side chain featuring a nitrogen-containing heterocyclic ring, and alendronate, characterized as a moderately potent N-BP[35]. The micromolar concentrations used in the experiments were based on published studies reporting the serum concentrations of these compounds in patients undergoing osteoporosis or bone cancer treatment after drug infusions[36–38]. For the experiments, human endothelial cells were cultured for six days with 5 µM alendronate or 1 µM zoledronate. We have previously shown that in endothelial cells at these concentrations, alendronate and zoledronate have no effect on cell viability but lead to a similarly significant reduction (by ~ 30%) in the cellular CoQ content[19]. Higher concentrations (from 7.5 µM for alendronate to 2.5 µM for zoledronate) significantly reduce endothelial cell viability and strongly (by ~ 60%) reduce the cellular CoQ content[19].

Total and reduced mtCoQ levels were significantly decreased, and SOD2 levels were increased in the endothelial mitochondria of N-BP-exposed cells

To date, the impact of N-BPs on mtCoQ levels has not been studied extensively. We observed a 45–50% decrease in total mtCoQ content ($mtCoQH_2$ + mtCoQ oxidized) in mitochondria isolated from endothelial cells cultured with 5 µM alendronate or 1 µM zoledronate compared with the mitochondria of control cells (Fig. 1a). Under fully oxidizing conditions (without respiratory mtCoQ-reducing substrates), we also measured the pool of reduced mtCoQ ($mtCoQH_2$), which is not oxidizable by the respiratory chain and may act as an antioxidant pool[1]. The $mtCoQH_2$ pool constituted ~ 12% of the total mtCoQ pool in the mitochondria of control cells and was not observed in the mitochondria of N-BP-treated cells. Furthermore, a significant ~ 20% decrease in the level of CoQ10B, which is required for mtCoQ function in the respiratory chain, was observed in the mitochondria of N-BP-treated cells (Fig. 1b). The disappearance of the reduced pool of mtCoQ ($mtCoQH_2$) was accompanied by a 10–20% increase in the antioxidant enzyme SOD2 level.

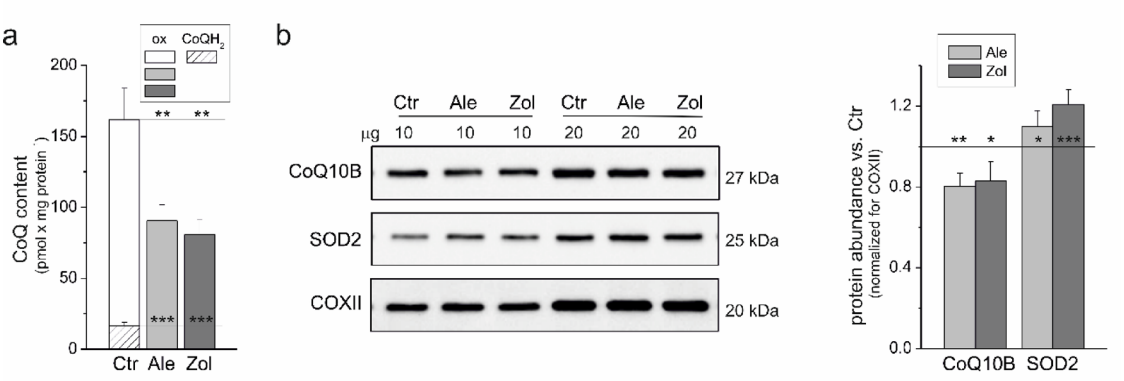

Fig. 1. (**a**) mtCoQ content and (**b**) protein abundance of CoQ10B and SOD2 in mitochondria isolated from endothelial cells exposed for six days to 5 µM alendronate (Ale) or 1 µM zoledronate (Zol) compared with the mitochondria of control cells (Ctr). (**a**) The total ($mtCoQH_2$ + mtCoQox), reduced ($mtCoQH_2$), and oxidized (mtCoQox) CoQ pools were determined under fully oxidizing conditions (without respiratory mtCoQ-reducing substrates). Mean ± SD; $n = 8$; statistics: one-way ANOVA, $P < 0.001$ (***); $P < 0.01$ (**) relative to control mitochondria (horizontal lines). (**b**) Analyses of protein abundance accompanied by representative immunoblots. Loading control: COXII. The original immunoblots are shown in Fig. S1 (Supplementary Information). Mean ± SD; $n = 5$; statistics: KW ANOVA, $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*) relative to control mitochondria (horizontal lines).

$mtCoQH_2$ is not only an important antioxidant but also a key electron carrier in the mitochondrial respiratory chain. Therefore, we next examined whether the decrease in mtCoQ observed after endothelial cell exposure to N-BPs influences mitochondrial respiration.

Chronic six-day exposure of endothelial cells to N-BPs leads to an overall reduction in the oxidation of respiratory substrates in their mitochondria, except for increased fatty acid oxidation

We assessed the effects of treating endothelial cells with 5 µM alendronate or 1 µM zoledronate on mitochondrial respiratory function by measuring the maximal respiratory rate of isolated endothelial mitochondria via various respiratory substrates (Fig. 2a). Under uncoupling conditions, maximal oxidation of the weakest reducing substrate tested, palmitoylcarnitine, was increased by ~ 20% in the mitochondria of N-BP-treated endothelial cells compared with those of control cells. The enhanced oxidation of this substrate was accompanied by a similar increase in ACADS protein, the enzyme that catalyzes the initial step of fatty acid β-oxidation (Fig. 2b). This observation may indicate an increased share of fatty acids as energy fuels in oxidative metabolism in endothelial cells treated with N-BPs.

However, in the mitochondria of endothelial cells treated with alendronate or zoledronate, maximal glutamate oxidation and protein abundance of GDH, the enzyme responsible for the conversion of glutamate to α-ketoglutarate, which enters the tricarboxylic acid (TCA) cycle for oxidation, decreased slightly by ~ 13% relative to that in the mitochondria of control untreated cells (Fig. 2). Moreover, a significant reduction in the maximal oxidation of strongly reducing substrates (i.e., the CI substrate malate [~ 17%], the CII substrate succinate [~ 25%], and their mixture [~ 21%]) was observed (Fig. 2a). Therefore, we next examined the impact of these changes on mitochondrial ATP synthesis efficiency and ΔΨ.

Respiratory rates, ΔΨ, and ATP synthesis efficiency during the oxidation of CI and CII substrates are reduced in the mitochondria of N-BP-treated endothelial cells

The functional bioenergetics parameters of endothelial mitochondria were compared during the oxidation of succinate alone (with rotenone), malate alone, and their mixture under non-phosphorylating and phosphorylating conditions. For all the tested substrates (Table 1), the chronic exposure of endothelial cells to alendronate or zoledronate decreased mitochondrial coupling parameters, the ADP/O ratio (by 13–16%), and the respiratory control ratios (by 13–19%), thus decreasing mitochondrial OXPHOS efficiency. Consequently, the ADP phosphorylation rate was significantly lower, albeit less significantly for malate alone (~30%) and most significantly for succinate alone (~40%), in the mitochondria of N-BP-treated cells compared to that in the mitochondria of control cells. These results indicate significantly reduced ATP synthesis in the mitochondria of N-BP-treated endothelial cells.

In contrast to non-phosphorylating conditions, under phosphorylating conditions, the respiratory rates with malate in the mitochondria of N-BP-treated cells were ~ 20% lower than those in the control cell mitochondria (Fig. 3a). Moreover, during succinate oxidation, reductions of ~ 17% and ~ 30% were observed under non-phosphorylating and phosphorylating conditions, respectively. Compared with that in the mitochondria of control cells, oxidation of the mixture of both substrates was also reduced in the mitochondria of N-BP-treated cells, and was less significant under non-phosphorylating (~ 13%) than phosphorylating (~ 26%) conditions. Additionally, under non-phosphorylating conditions, decreases in the respiration of CI and CII substrates

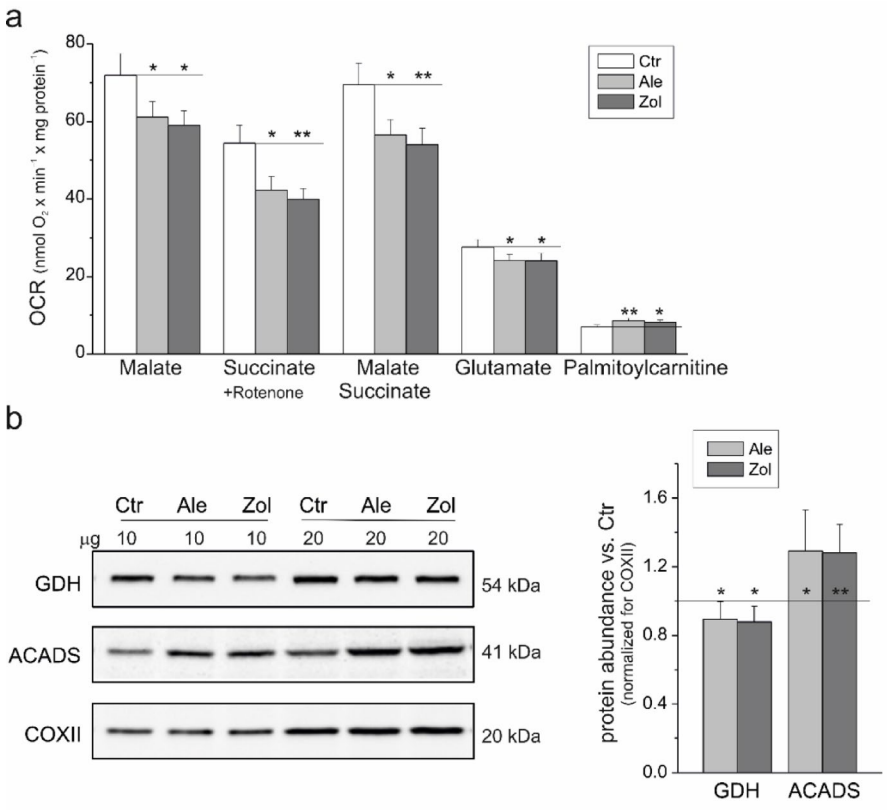

Fig. 2. (**a**) Maximal respiratory chain activity (uncoupled respiration) with different substrates and (**b**) ACADS and GDH protein levels in mitochondria isolated from endothelial cells cultured with 5 µM alendronate (Ale) or 1 µM zoledronate (Zol) compared with the mitochondria of control cells (Ctr). (**a**) Uncoupled respiration in the presence of 0.8 µM FCCP; OCR, oxygen consumption rate. Mean ± SD; $n = 8$; statistics: one-way ANOVA, $P < 0.01$ (**); $P < 0.05$ (*) relative to control mitochondria (horizontal lines). (**b**) Analyses of protein abundance accompanied by representative immunoblots. Loading control: COXII. The original immunoblots can be found in Fig. S2 (Supplementary Information). Mean ± SD; $n = 6$; $P < 0.01$ (**); P 0.05 (*) relative to control mitochondria (horizontal lines).

	Malate			Succinate + Rotenone			Succinate + Malate		
	Ctr	Ale	Zol	Ctr	Ale	Zol	Ctr	Ale	Zol
RCR	4.28 ± 0.35	3.64 ± 0.31*	3.63 ± 0.34*	2.74 ± 0.23	2.30 ± 0.20*	2.31 ± 0.19*	3.46 ± 0.26	2.99 ± 0.23*	2.88 ± 0.23*
ADP/O	2.36 ± 0.18	1.99 ± 0.12*	2.05 ± 0.15*	1.32 ± 0.09	1.07 ± 0.08*	1.07 ± 0.08*	2.01 ± 0.17	1.67 ± 0.11*	1.69 ± 0.12*
Phosphorylation rate	163 ± 13	110 ± 8***	110 ± 10***	68.6 ± 5.6	39.6 ± 3.3***	38.3 ± 3.0***	135 ± 12	82.1 ± 6.1***	82.4 ± 6.6***

Table 1. Mitochondrial coupling parameters—the respiratory control ratio (RCR), the ADP/O ratio, and the rate of ADP phosphorylation (phosphorylating respiration × ADP/O)—in the mitochondria of control (Ctr) and alendronate (Ale)- or zoledronate (Zol)-treated endothelial cells. The phosphorylation rate is expressed in nmol ADP × min^{-1} × mg $protein^{-1}$. Mean ± SD ($n = 8$); statistics: one-way ANOVA, *$P < 0.05$, *** $P < 0.001$ compared with control mitochondria.

were accompanied by decreases in ΔΨ (the largest during succinate oxidation; Fig. 3b). Under phosphorylating conditions, a statistically significant reduction in ΔΨ was observed only during the oxidation of succinate alone in the mitochondria of N-BP-treated cells compared with the mitochondria of control cells.

Overall, the mitochondria of endothelial cells cultured in the presence of alendronate or zoledronate presented a lower respiratory rate, ΔΨ, and OXPHOS efficiency than did the mitochondria of control cells. Notably, the observed changes were more pronounced during the oxidation of the CII substrate (succinate) than during the oxidation of the CI substrate (malate) when each substrate was oxidized individually.

$mtCoQH_2$ is not only an important antioxidant but also plays a role in generating mtROS via the respiratory chain. Therefore, we investigated whether the decrease in mtCoQ observed after the exposure of endothelial cells to N-BPs affects the reduction level of mtCoQ and, thus, the production of mtROS.

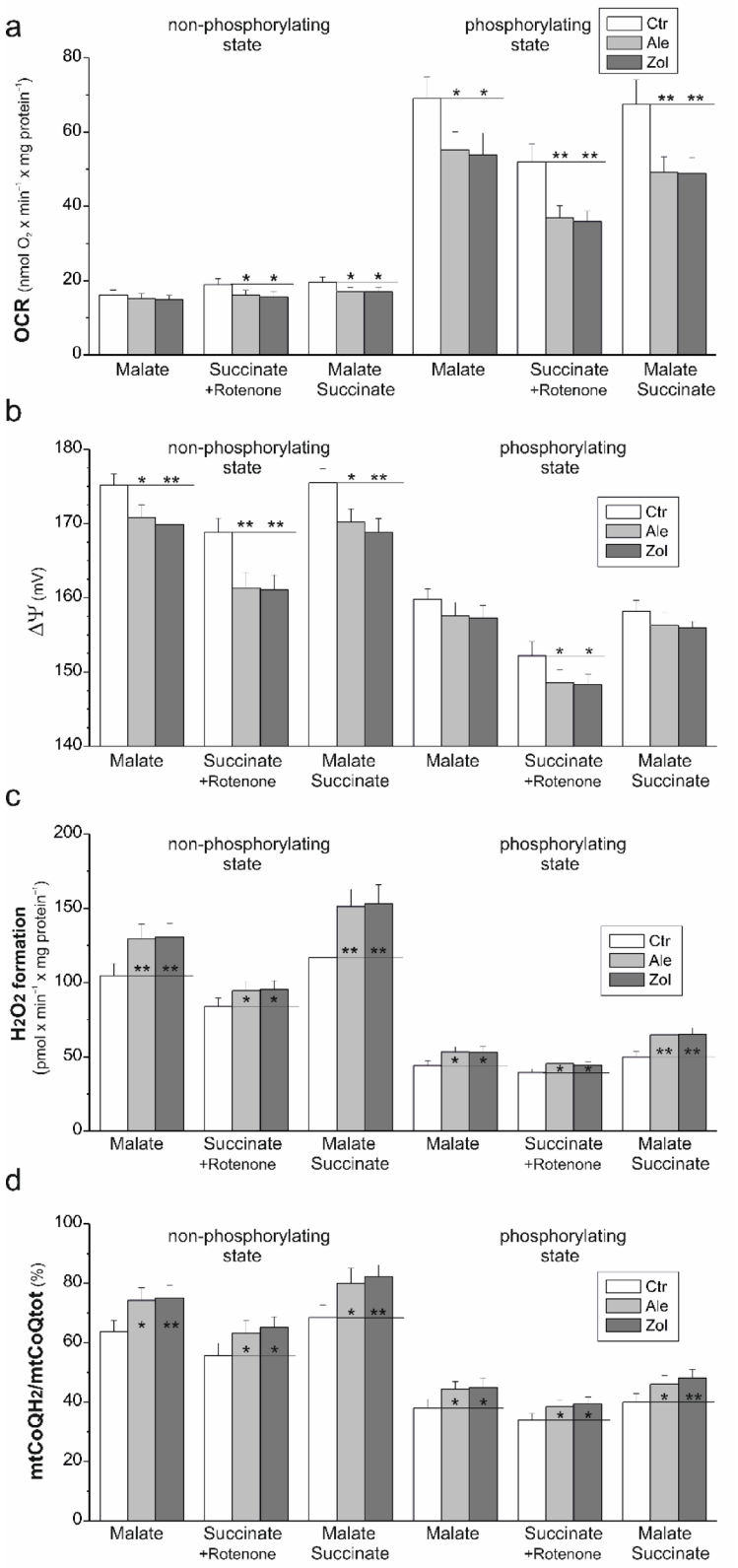

Fig. 3. Functional parameters of mitochondria isolated from control (Ctr) and alendronate (Ale)- or zoledronate (Zol)-treated endothelial cells during the oxidation of malate, succinate (with rotenone), and a mixture of malate and succinate under non-phosphorylating and phosphorylating conditions. (**a**) Oxygen consumption rate (OCR). (**b**) ΔΨ. (**c**) H_2O_2 production rate. (**d**) mtCoQ reduction level ($mtCoQH_2$/mtCoQtot). Mean ± SD; $n = 6–8$; statistics: one-way ANOVA, $P < 0.01$ (**); $P < 0.05$ (*) relative to control mitochondria (horizontal lines).

Chronic exposure of endothelial cells to N-BPs increases H_2O_2 production in mitochondria due to increased mtCoQ reduction level

In the mitochondria of N-BP-exposed endothelial cells relative to the mitochondria of control cells, H_2O_2 production was consistently greater under both respiratory conditions, regardless of the substrate (malate alone, succinate alone, or a combination of malate and succinate) (Fig. 3c). A comparison of H_2O_2 production during the oxidation of malate alone and of succinate alone revealed that these increases were more pronounced for the CI substrate (malate; ~22–25%) than for the CII substrate (succinate; ~13–15%). Moreover, in the mitochondria of N-BP-treated cells, the level of mtCoQ reduction consistently increased under both non-phosphorylating and phosphorylating conditions, regardless of the substrate (Fig. 3d). Likewise, these increases were more prominent during the CI substrate (~18%) versus CII substrate (~13%) oxidation.

Regarding the oxidation of the tested substrates under non-phosphorylating and phosphorylating conditions by the mitochondria of N-BP-treated and control cells, a single relationship was obtained between H_2O_2 production and the level of mtCoQ reduction (Fig. 4a), confirming the direct dependence of mtROS generation on the level of mtCoQ reduction[39]. The relationship between ΔΨ and the mtCoQ reduction level obtained for the mitochondria of N-BP-treated cells shifted relative to the mitochondria of control cells to values with higher H_2O_2 production and lower ΔΨ (Fig. 4b), indicating inhibition of the $mtCoQH_2$-oxidizing pathway. To investigate further, we analyzed the activities of CIII and CIV. In the mitochondria of N-BP-treated cells, CIII activity was ~ 20% lower than that in the mitochondria of control cells, whereas CIV activity was unaffected (Fig. 4c). However, in the mitochondria of N-BP-treated cells, the level of reduction of cytochromes $a + a_3$, components of CIV, was ~ 17% lower than that in the mitochondria of control cells (Fig. 4d). The latter result indicates that in the mitochondria of endothelial cells treated with N-BPs, these mevalonate pathway inhibitors may decrease the level of heme *a*, another product of the pathway in addition to CoQ. However, this result requires further investigation.

Our results indicate that the overall increase in mtROS production is due to the increased level of mtCoQ reduction ($mtCoQH_2$/mtCoQtot) resulting from a significant decrease in the size of the mtCoQ pool in the mitochondria of N-BP-treated endothelial cells. To understand why, in the mitochondria of N-BP-treated endothelial cells, oxidation of the CII substrate caused greater decreases in respiration and ΔΨ but smaller increases in mtROS production and mtCoQ reduction level compared with oxidation of the CI substrate, we

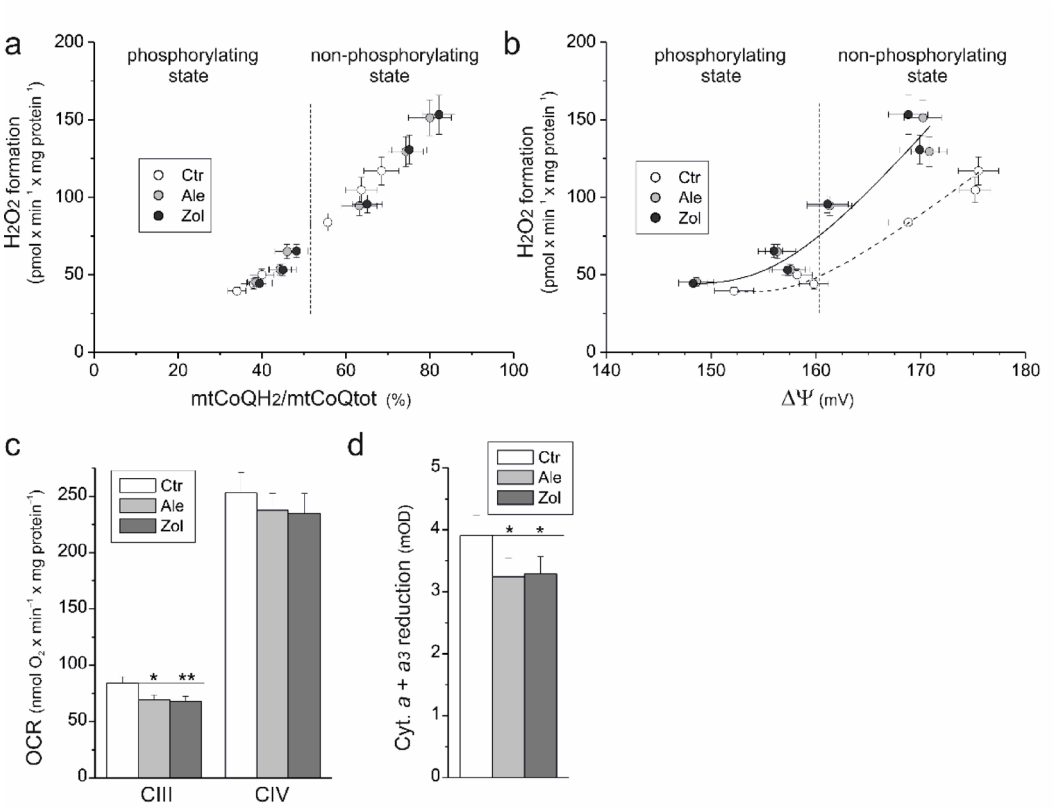

Fig. 4. Relationships between (**a**) H_2O_2 production and the mtCoQ reduction level and (**b**) H_2O_2 production and ΔΨ of the mitochondria isolated from control (Ctr) and alendronate (Ale)- or zoledronate (Zol)-treated endothelial cells during succinate (with rotenone), malate, and a mixture of succinate and malate oxidation under non-phosphorylating and phosphorylating conditions. (**c**) Maximal CIII activity and CIV activity. (**d**) Reduction of cytochromes $a + a_3$. Mean ± SD; $n = 4–6$; statistics: one-way ANOVA, $P < 0.01$ (**); $P < 0.05$ (*) relative to control mitochondria (horizontal lines; **c**, **d**).

further examined the N-BP-induced changes in the activity and levels of individual components of the OXPHOS system and its molecular organization.

N-BPs change the molecular organization of the OXPHOS system in the mitochondria of endothelial cells, downregulating CIII and CIV in the $CIII_2$ + CIV supercomplex and CII, $CIII_2$, and CV

Immunodetection of OXPHOS components revealed an ~ 20% reduction in the abundance of CII (subunit SDHB), CIII (subunit Core 2), and ATP synthase (subunit α) in the mitochondria of N-BP-treated endothelial cells (Fig. 5a). In contrast, the protein levels of CI (subunit NDUFB8) and CIV (subunit COXII) remained unchanged (Fig. 5a). Furthermore, BN-PAGE followed by in-gel activity assays revealed that CII and CV (ATP synthase) activities decreased after exposing endothelial cells to alendronate or zoledronate (Fig. 5b). In contrast, CI activities in all CI-associated supercomplexes remained unchanged (Fig. 5b).

In the mitochondria of N-BP-treated endothelial cells, while the protein levels of CI + CIII-associated supercomplexes ($I + III_2 + IV_{(n)}$ and $I + III_2$) did not differ from those in the mitochondria of control cells, the levels of the III_2 + IV supercomplex and III_2 dimer decreased (Fig. 5b). These observations are consistent with the decreased level of CIII (subunit Core 2) observed via SDS-PAGE (Fig. 5a) and with the decreased CIII activity, measured during the oxidation of duroquinol (Fig. 4c) in intact mitochondria of N-BP-treated endothelial cells. Furthermore, the in-gel activity of CIV in the III_2 + IV supercomplex decreased in the mitochondria of N-BP-treated endothelial cells, whereas the activity of other CIV-associated supercomplexes/complexes and the protein level of CIV (COXII subunit) did not change (Fig. 5). However, in intact mitochondria of N-BP-treated endothelial cells, CIV activity remained unchanged (Fig. 4c), although the levels of the III_2 + IV supercomplex (Fig. 5b) and cytochromes $a + a_3$ (Fig. 4d) also decreased.

These results indicate that chronic exposure to N-BPs, which leads to a significant decrease in the level of mtCoQ in endothelial mitochondria, causes a rearrangement of the molecular organization of the respiratory

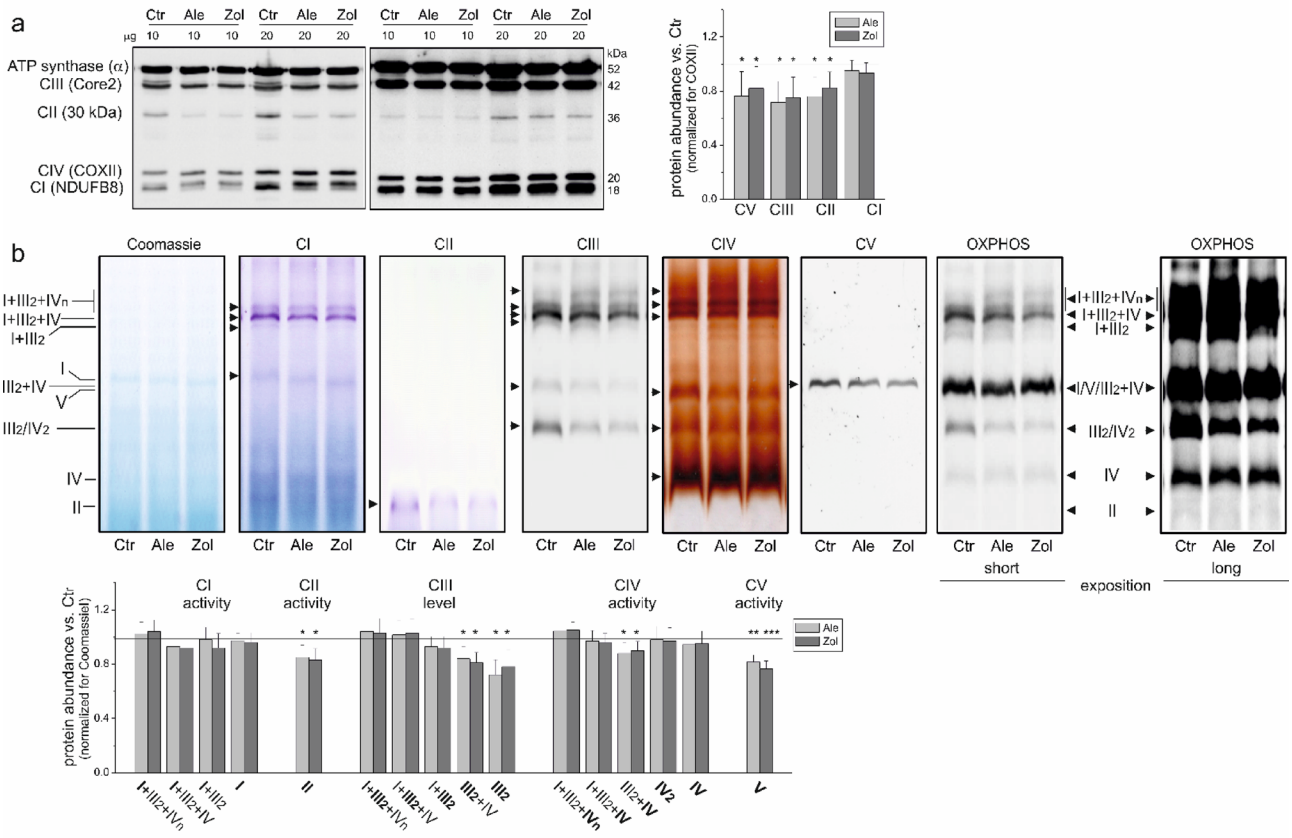

Fig. 5. (**a**) OXPHOS complexes and (**b**) supercomplexes in the mitochondria isolated from control (Ctr) and alendronate (Ale)- or zoledronate (Zol)-treated endothelial cells. (**a**) Representative immunoblots and analysis of protein abundance. Mean ± SD; $n = 6$; statistics: one-way ANOVA, $P < 0.05$ (*) relative to control mitochondria. (**b**) Representative BN-PAGE showing OXPHOS supercomplexes and complexes, and analysis of changes in in-gel activity or protein levels. Coomassie staining, CI and CII in-gel activity, CIII immunoblotting, CIV and CV in-gel activity, and total OXPHOS immunoblotting (short and long exposition) are shown in sequence. Mean ± SD; $n = 4$; statistics: KW ANOVA, $P < 0.001$ (***); $P < 0.01$ (**); $P < 0.05$ (*) relative to control mitochondria. The gels and original immunoblots are shown in Fig. S3 (Supplementary Information). CI, CII, CIII, CIV, respiratory chain complexes; CV, F_0F_1 ATP synthase.

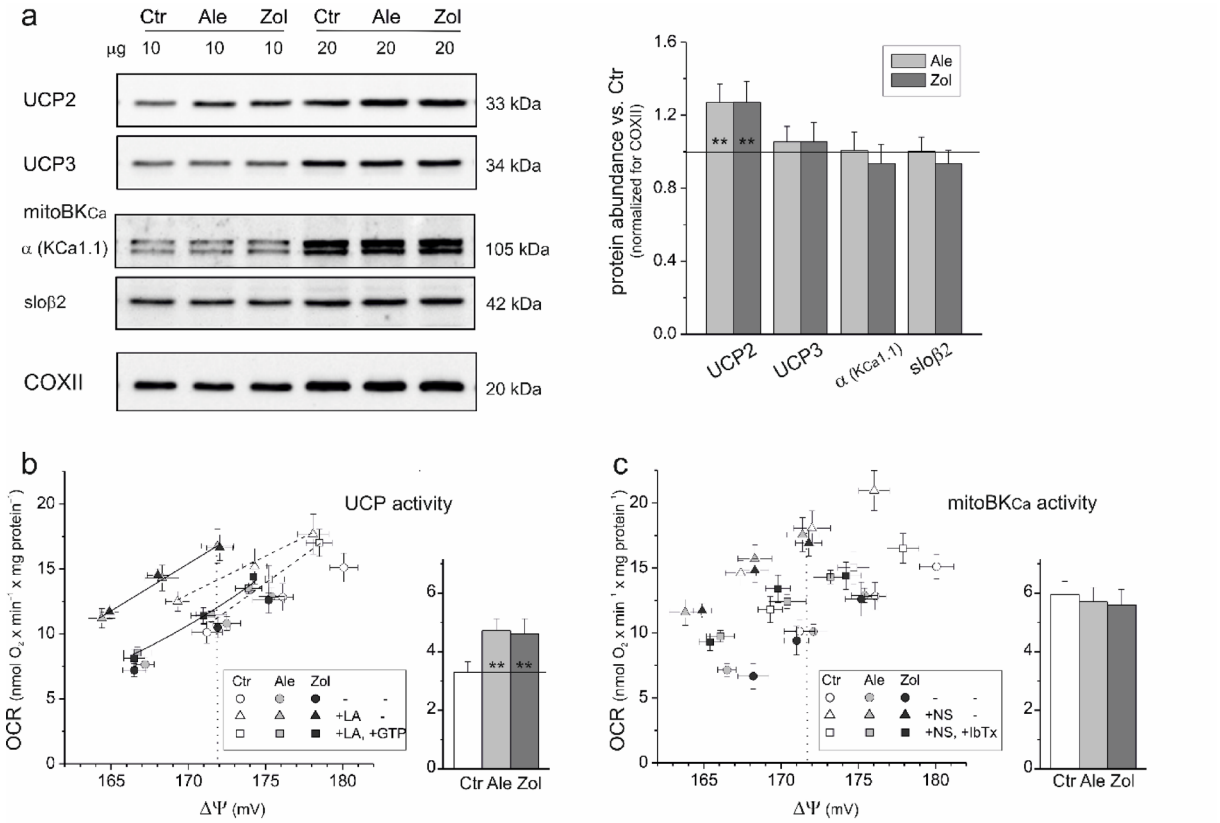

Fig. 6. (**a**) Protein levels and (**b**) activities of energy-dissipating systems, UCP and mitoBK$_{Ca}$, in the mitochondria of control (Ctr) and alendronate (Ale)- or zoledronate (Zol)-treated endothelial cells. (**a**) Analyses of protein abundance accompanied by representative immunoblots. Mean ± SD; $n = 6$; statistics: one-way ANOVA, $P < 0.01$ (**) relative to control mitochondria. Subunits of mitoBK$_{Ca}$: α (K$_{Ca}$1.1), pore α subunit K$_{Ca}$1.1, and sloβ2, auxiliary sloβ2 subunit. The original immunoblots can be found in Figs. S4, S5 (Supplementary Information). (**b**) Linoleic acid (LA)-induced GTP-inhibited UCP-mediated H^+ leakage (UCP activity). **c**, NS11021 (NS)-induced IbTx-inhibited mitoBK$_{Ca}$ activity. (**b**, **c**) Relationships between the oxygen consumption rate (OCR) and ΔΨ for rotenone + malonate titration during the oxidation of a malate-succinate mixture under non-phosphorylating conditions. UCP and mitoBK$_{Ca}$ activity were determined at the same ΔΨ (~ 172 mV). Mean ± SD; $n = 4$; statistics: KW ANOVA, $P < 0.01$ (**) relative to control mitochondria.

chain supercomplexes (i.e., a decrease in the level of the III_2 + IV supercomplex with an accompanying decrease in the protein level and activity of CII, CIII, and CV). These changes may lead to more effective use of deficient mtCoQ in the mitochondria of endothelial cells treated with N-BPs.

In N-BP-treated endothelial cells, mitochondrial UCP2 expression is increased

The question arises whether, owing to the increased mtROS production by the mitochondria of endothelial cells treated with alendronate or zoledronate (Fig. 3c), N-BPs affect the mitochondrial energy-dissipating systems, UCP and mitoBK$_{Ca}$, which may act as antioxidant systems by increasing electron flow in the respiratory chain[33,40,41]. UCP2 expression was ~ 27% higher in the mitochondria of N-BP-treated endothelial cells than in the mitochondria of control cells, whereas UCP3 expression was unchanged (Fig. 6a). The protein levels of the mitoBK$_{Ca}$ subunits, including the pore α subunit and regulatory sloβ2 subunit (found in endothelial mitochondria[42] were also unaffected in the mitochondria of N-BP-treated cells (Fig. 6a).

Moreover, the flux–force kinetics assay revealed increased fatty acid-induced GTP-inhibited UCP activity (~ 40%) and unchanged NS11021-induced IbTx-inhibited mitoBK$_{Ca}$ activity (Fig. 6b and c) in the mitochondria of N-BP-treated cells compared with the mitochondria of control cells, confirming the immunodetection results (Fig. 6a). The increase in UCP activity can be linked to UCP2, as its protein levels were elevated. When the activity of mtCoQ-reducing dehydrogenases (CI and CII) was reduced (by titration with rotenone and malonate), ΔΨ depolarization was accompanied by increased sensitivity of UCP activity to GTP. These results support that lower mtCoQ reduction enhances UCP activity inhibition by purine nucleotides[40,43].

These findings indicate that the mitochondria of N-BP-exposed endothelial cells exhibit higher levels of UCP2 expression and activity than the mitochondria of control cells, which may reduce mtROS production under conditions of mtCoQ deficiency.

Discussion

We have previously shown that 5 μM alendronate and 1 μM zoledronate cause a similar decrease in the cellular level of CoQ in endothelial cells without affecting cell viability[19]. The effects of such concentrations of these N-BPs on the bioenergetic function of endothelial mitochondria were also similar in the present study.

A comparison of mitochondria isolated from human endothelial cells cultured for six days with or without 5 μM alendronate or 1 μM zoledronate revealed a significant 45–50% decrease in mtCoQ content (Fig. 1a) caused by these inhibitors of a crucial enzyme of the mevalonate pathway. Thus, inhibition of the mevalonate pathway by N-BPs, which disrupts the synthesis of the isoprenoid chain necessary for CoQ biosynthesis[12,13], leads to reduced mtCoQ levels. Interestingly, the observed decrease in CoQ10B levels (Fig. 1b) also indicates potential changes in the CoQ biosynthetic pathway downstream of the mevalonate pathway[44]. However, further analyses are necessary to fully elucidate the regulatory mechanisms of the CoQ biosynthetic pathway under N-BP exposure, including other CoQ proteins essential for CoQ biosynthesis[44] at the transcriptional and posttranscriptional levels.

This study is the first to demonstrate an N-BP-induced decrease in mtCoQ levels in mitochondria. There is also little research on the effects of N-BPs on cellular CoQ levels. Plasma CoQ levels are decreased in postmenopausal women treated with N-BPs[18]. Our previous studies revealed an ~ 30% decrease in total CoQ levels (i.e., CoQ from all membranes) in endothelial cells cultured with alendronate or zoledronate[19]. The current study revealed that in the mitochondria of N-BP-treated endothelial cells, mtCoQ deficiency was accompanied by the loss of the pool of $mtCoQH_2$, which has an antioxidant function, and accounts for ~ 12% of the total mtCoQ pool in the mitochondria of untreated endothelial cells (Fig. 1a). The N-BP-induced loss of the $mtCoQH_2$ antioxidant pool was accompanied by increased levels of the antioxidant enzyme SOD2 and increased UCP2 levels and activity (Figs. 1b and 6a and b). Our previous studies demonstrated that UCP2 might play a physiological role in reducing mtROS formation during conditions of heightened oxidative stress in endothelial cells, such as exposure to high levels of glucose[45] and palmitic acid[46], and to statins, which, like N-BPs, block the mevalonate pathway[15].

The N-BP-induced 45–50% decrease in mtCoQ content (Fig. 1a) indicates a significantly lower availability of mtCoQ in the mitochondrial membrane, which may strongly affect the efficiency of the respiratory chain and ATP synthesis and impact mtROS formation, potentially affecting overall cellular metabolism. In endothelial mitochondria, N-BPs led to an overall reduction in the maximal oxidation of respiratory substrates (malate, succinate, and glutamate), whereas the oxidation of the least oxidized palmitoylcarnitine increased (Fig. 2). These findings suggest that N-BPs reduce the capacity of endothelial mitochondria to metabolize intermediates effectively in the TCA cycle. Furthermore, endothelial cells may adapt to reduced energy availability from glucose or amino acid oxidation by relying more on fat-derived energy sources. Our results show that N-BPs modulate ACADS, an enzyme involved in fatty acid oxidation, by increasing its levels (Fig. 2b). Previous studies have shown that endothelial cells exposed to N-BPs are less able to efficiently utilize glucose and amino acid substrates but demonstrate increased fatty acid oxidation[19]. In the mitochondria of N-BP-treated cells, decreased substrate flux into the electron transport chain leads to decreased ATP production, as indicated by a decreased ADP/O ratio, ADP phosphorylation rate (Table 1), and ATP synthase activity level (Fig. 5b). Fatty acids provide more ATP per molecule than carbohydrates do; thus, cells may attempt to optimize energy production under N-BP exposure by switching to a more energy-rich fuel source. Interestingly, the significant reduction in the mtCoQ pool (Fig. 1a) in the mitochondria of N-BP-treated cells was accompanied by a decrease in the activity and protein level of CII, not CI (Fig. 5). Oxidation of the CI substrate provides more ATP than does oxidation of the CII substrate (higher rate of ADP phosphorylation; Table 1). Thus, mitochondria may attempt to maintain energy production under N-BP exposure by limiting only the inflow of electrons from CII, not CI, to the diminished mtCoQ pool.

The decrease in the mtCoQ pool in mitochondria from N-BP-exposed endothelial cells resulted in a decline not only in mtCoQ-reducing CII (Fig. 5) but also in the activity and level of CIII that oxidizes QH_2 (Figs. 4c and 5). However, the decrease in the maximal activity of CIII in intact mitochondria was accompanied by rearrangements at the organizational level of its supercomplexes. The CIII protein level and the CI activity of the CI + CIII-related supercomplexes (I + III_2 + IV(n) and I + III_2) did not change, whereas the levels of the III_2 + IV supercomplex and the III_2 dimer decreased (Fig. 5b). These changes may serve to more effectively use the deficient mtCoQ and maintain electron flow through the CI + CIII + CIV pathway in the mitochondria of N-BP-treated endothelial cells. Previous studies have shown that statin- or hypoxia-induced mtCoQ deficiency also causes a specific rearrangement of the molecular organization of OXPHOS system components in endothelial mitochondria[15,33].

Our results indicate that in the mitochondria of cells treated with N-BPs, these mevalonate pathway inhibitors may decrease the level of *a*-heme (cytochromes $a + a_3$) (Fig. 4d), the prosthetic group of CIV. However, further studies are needed to investigate whether inhibition of the mevalonate pathway by N-BPs could impair the synthesis of the isoprenoid chain required for the biosynthesis of heme *a*, a key component of mitochondrial COX (CIV), potentially leading to reduced cytochrome *a* levels and impaired mitochondrial respiratory function. However, in our study, despite reduced *a*-heme levels in the CIV (Fig. 4d), no effect on the maximal activity of this complex was observed in the intact mitochondria of N-BP-treated endothelial cells compared with that of the mitochondria of control cells (Fig. 4c). Only the in-gel activity of CIV in the III_2 + IV supercomplex decreased in the mitochondria of N-BP-treated cells (Fig. 5b). The maximal activity of CIV (~ 250 nmol O_2 × min^{-1} × mg $protein^{-1}$; Fig. 4c) is not a factor limiting the flow of electrons in the respiratory chain in endothelial mitochondria, because with the activity of both CI and CII dehydrogenases, the maximal activity of the chain is much lower (~ 70 nmol O_2 × min^{-1} × mg $protein^{-1}$; Fig. 3a).

Our findings demonstrate that prolonged exposure of endothelial cells to alendronate or zoledronate modulates the activity and composition of the mitochondrial respiratory chain and slows OXPHOS. Moreover,

the significant N-BP-induced ~ 45–50% decrease in mtCoQ content (Fig. 1a) resulted in increased mtCoQ reduction ($mtCoQH_2$/mtCoQtot) under both phosphorylating and non-phosphorylating conditions (Fig. 3d), resulting in increased mtROS formation (Fig. 3c). The decreases in the mtCoQ pool and the activity of the $mtCoQH_2$-oxidizing pathway (CIII + CIV) related to decreased CIII activity may account for the increased reduction in mtCoQ, resulting in a general increase in mtROS production in the mitochondria of N-BP-treated endothelial cells. The decrease in CII activity, along with the stable CI activity (Fig. 5), accounts for the more significant decreases in respiration and ΔΨ (Figs. 3a, b) and the less pronounced increases in mtROS production and mtCoQ reduction (Figs. 3c, d) observed during CII substrate oxidation than during CI substrate oxidation. Decreasing mtCoQ-reducing dehydrogenase (CII) activity decreases the level of mtCoQ reduction and therefore mtROS formation. Decreasing $mtCoQH_2$-oxidizing CIII activity (pronounced during phosphorylating respiration) increases the level of mtCoQ reduction and mtROS production. Moreover, the inhibition of the $mtCoQH_2$-oxidizing pathway is clearly indicated by the shift in the relationship between ΔΨ and the mtCoQ reduction level, obtained for the mitochondria of N-BP-treated cells relative to the mitochondria of control cells, to values with higher ROS production and lower ΔΨ (Fig. 4b). Thus, our results indicate that exposing endothelial cells to N-BPs alters mtCoQ redox homeostasis, a key factor modulating mtROS production[1,39]. The increased mtCoQ reduction level induced by N-BPs and, consequently, the increased mtROS production (Figs. 3c, d) were accompanied by elevated levels of the antioxidant enzymes SOD2 and UCP2 (Figs. 1 and 6). For the first time, our study correlates changes in mtCoQ content, mtCoQ reduction level, and mtROS production in the mitochondria of endothelial cells exposed to N-BPs.

The observed disruption of mtCoQ homeostasis by N-BPs has important implications for endothelial pathophysiology, particularly in the context of cardiovascular disease, in which mitochondrial dysfunction and oxidative stress contribute to the onset and progression of the disease[20–23,47,48]. Decreased $mtCoQH_2$ levels directly impair the mitochondrial antioxidant defense system, leading to increased ROS production, which may activate redox-sensitive signaling pathways involved in endothelial activation, inflammation, and apoptosis. These mechanisms are closely related to the initial stages of atherosclerosis and microcirculation dysfunction[20,49,50]. In addition, reduced expression of key respiratory complexes (CII, CIII, and CV) and remodeling of supercomplexes may contribute to bioenergetic failure and induce metabolic reprogramming in endothelial cells, favoring fatty acid oxidation over glucose metabolism, a phenotype observed in endothelial cells under conditions of chronic stress or aging[15,19,45,51–54]. Targeting these mitochondrial vulnerabilities, for example through CoQ supplementation, represents a promising therapeutic avenue. Further studies should aim to validate these mechanisms in in vivo models and clinical settings.

Although N-BP drugs are commonly used to treat osteoporosis and other bone diseases, evidence suggests that long-term N-BP or high-dose N-BP therapy may have unintended effects on extraskeletal tissues, including the endothelium[24–27]. Although the primary aim of our study was to analyze the bioenergetic effects of N-BPs on endothelial mitochondria, the disturbances in mitochondrial function and mtCoQ redox homeostasis we observed may have broader implications for cardiovascular health. We propose that N-BP-induced mitochondrial dysfunction, particularly through mtCoQ deficiency/depletion and the resulting increase in mtROS, may contribute to the development of endothelial dysfunction and increase the risk of cardiovascular disease especially in individuals receiving long-term N-BP therapy. However, while this study provides important mechanistic insights at the mitochondrial level, further research is needed to determine the clinical relevance of these findings. Further studies are needed to investigate the systemic cardiovascular effects of N-BPs in animal models or clinical populations to better elucidate the full extent of these effects and their clinical implications. Furthermore, future studies should evaluate whether supplementation with exogenous CoQ, particularly in its bioavailable form (e.g., mitochondria-targeted CoQ10), can counteract N-BP-induced mitochondrial dysfunction and protect endothelial function, confirming the role of mtCoQ deficiency in the observed mitochondrial dysfunction and redox imbalance.

Conclusions

Our study revealed that chronic exposure to N-BPs, which are commonly used to treat osteoporosis, significantly affects mitochondrial bioenergetic functions in human endothelial cells, primarily by dramatically reducing the mtCoQ content. In N-BP-treated endothelial cells, a significant decrease in mtCoQ content was accompanied by an overall decrease in mitochondrial respiratory activity and OXPHOS efficiency, as well as a more reduced redox state of mtCoQ, leading to increased mtROS production. Moreover, changes at the molecular organization level of the OXPHOS system and a greater share of fatty acid oxidation were observed. Understanding the N-BP-induced adaptation of mitochondrial bioenergetics associated with mtCoQ deficiency may shed light on the molecular mechanisms underlying potential N-BP-induced endothelial dysfunction. This study also provides information to support strategies to mitigate potential side effects, possibly through CoQ10 supplementation. Because mitochondria are potential sites of pharmacological intervention aimed at broadly understood cell protection against oxidative stress, our research may prove helpful in verifying or supplementing (e.g., through CoQ10 supplementation) existing N-BP therapies. This study highlights the importance of conducting further research on the effects of N-BPs on endothelial mitochondria and the overall energy metabolism of endothelial cells.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

A.B., L.G., and W.J. conceptualized and designed the study; A.B., L.G. and K.W. performed experiments: A.B.—all methods, K.W.—assistance, and L.G.—SDS-PAGE, BN-PAGE; A.B., L.G., and W.J. analyzed data; A.B., L.G., and W.J. wrote the manuscript and made manuscript revisions.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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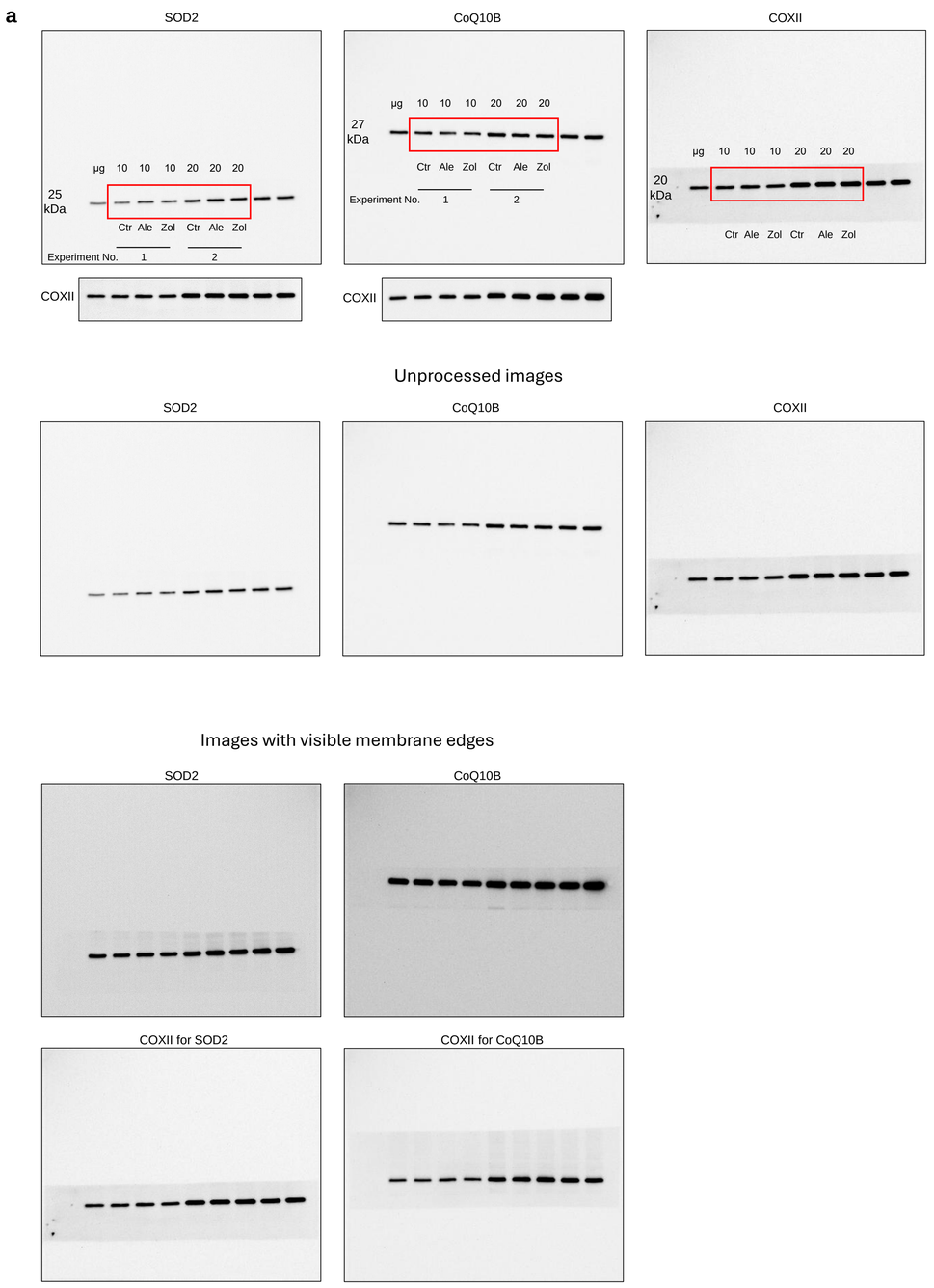
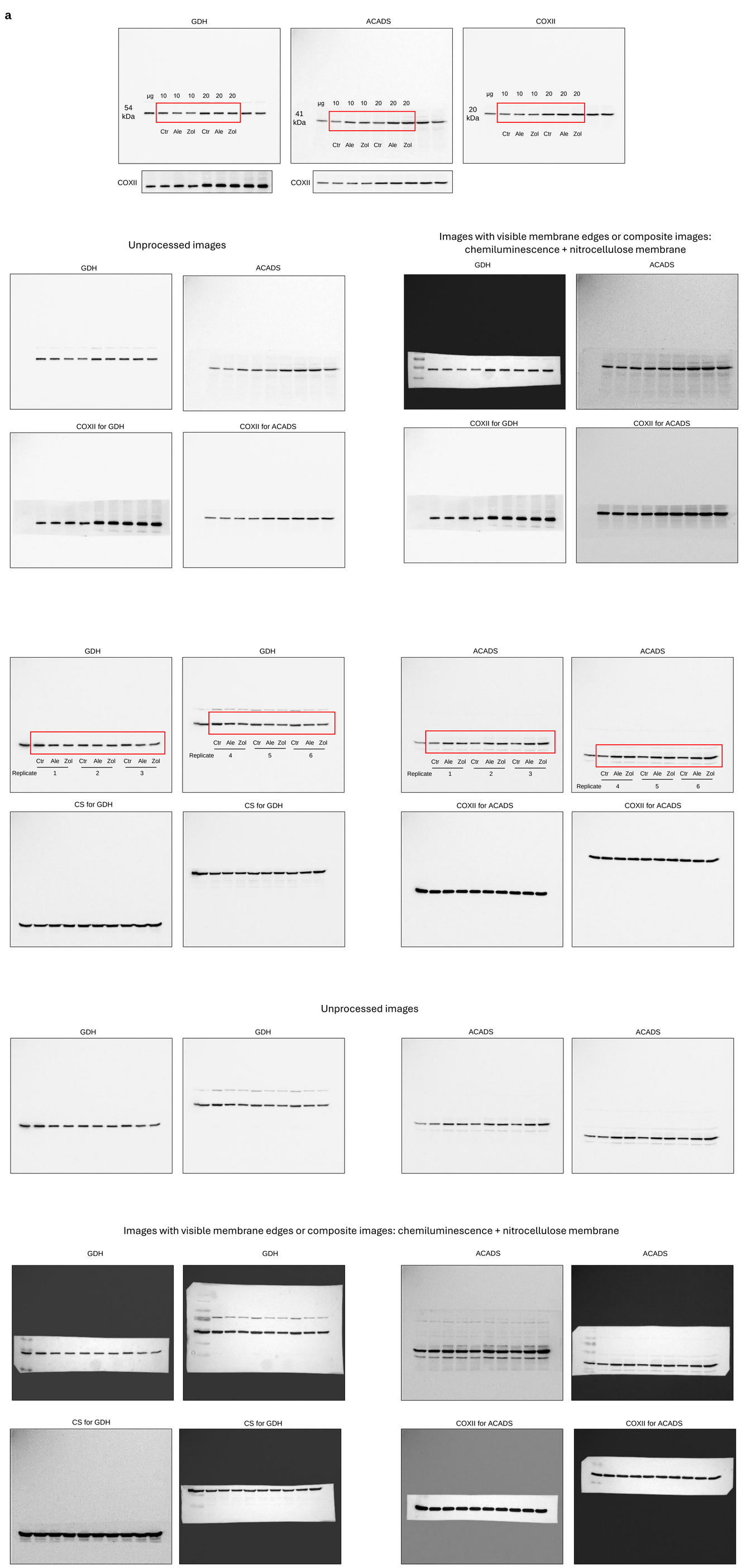
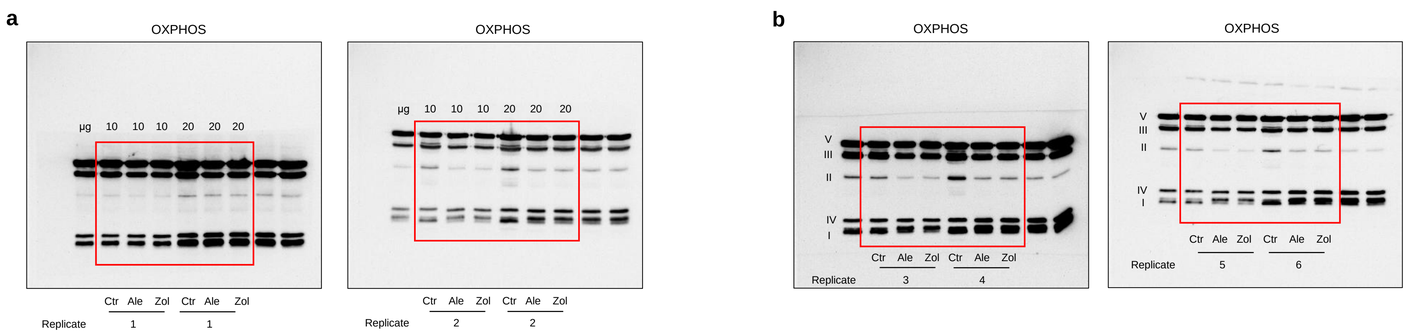


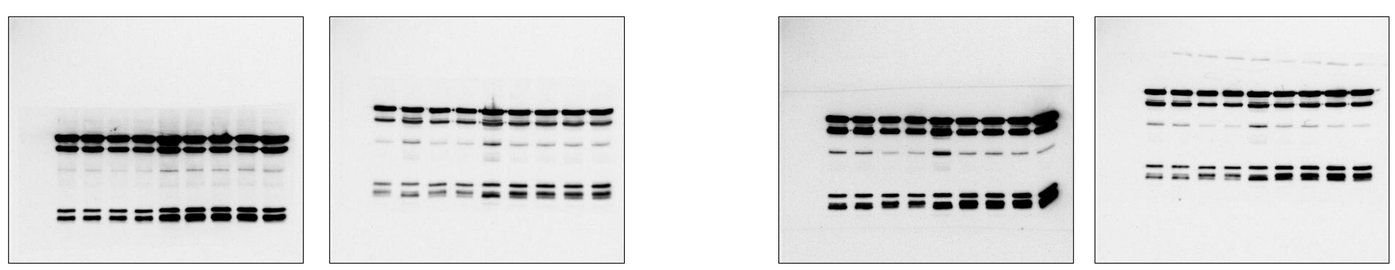
Supplementary Fig. S1. Uncropped images of blots shown in Fig. 1b (**a**) and images used for densitometric analysis (**b**). Ctr, control mitochondria; Ale, mitochondria of alendronate-treated cells; Zol, mitochondria of zoledronate-treated cells.



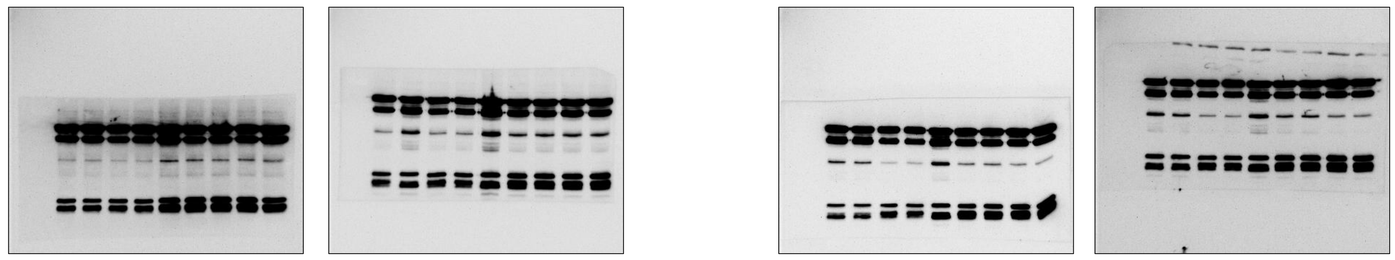
Supplementary Fig. S2. Original images of western blots used to prepare Fig. 2b (**a**) and blots used for densitometric analysis (**b**). Ctr, control mitochondria; Ale, mitochondria of alendronate-treated cells; Zol, mitochondria of zoledronate-treated cells.



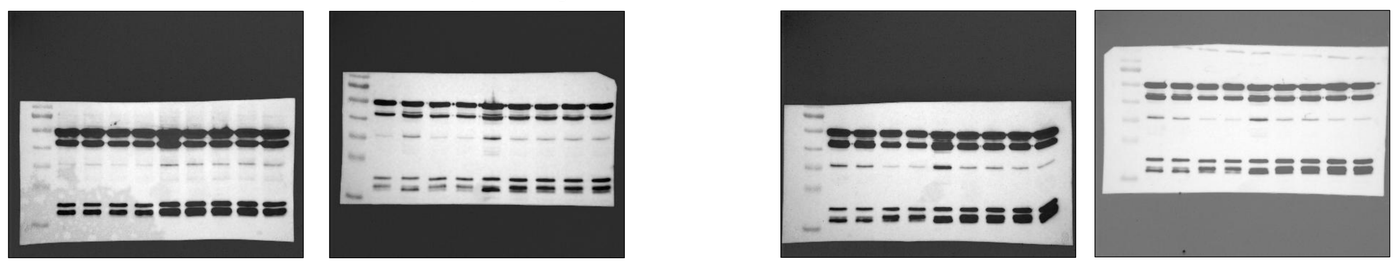
Unprocessed images

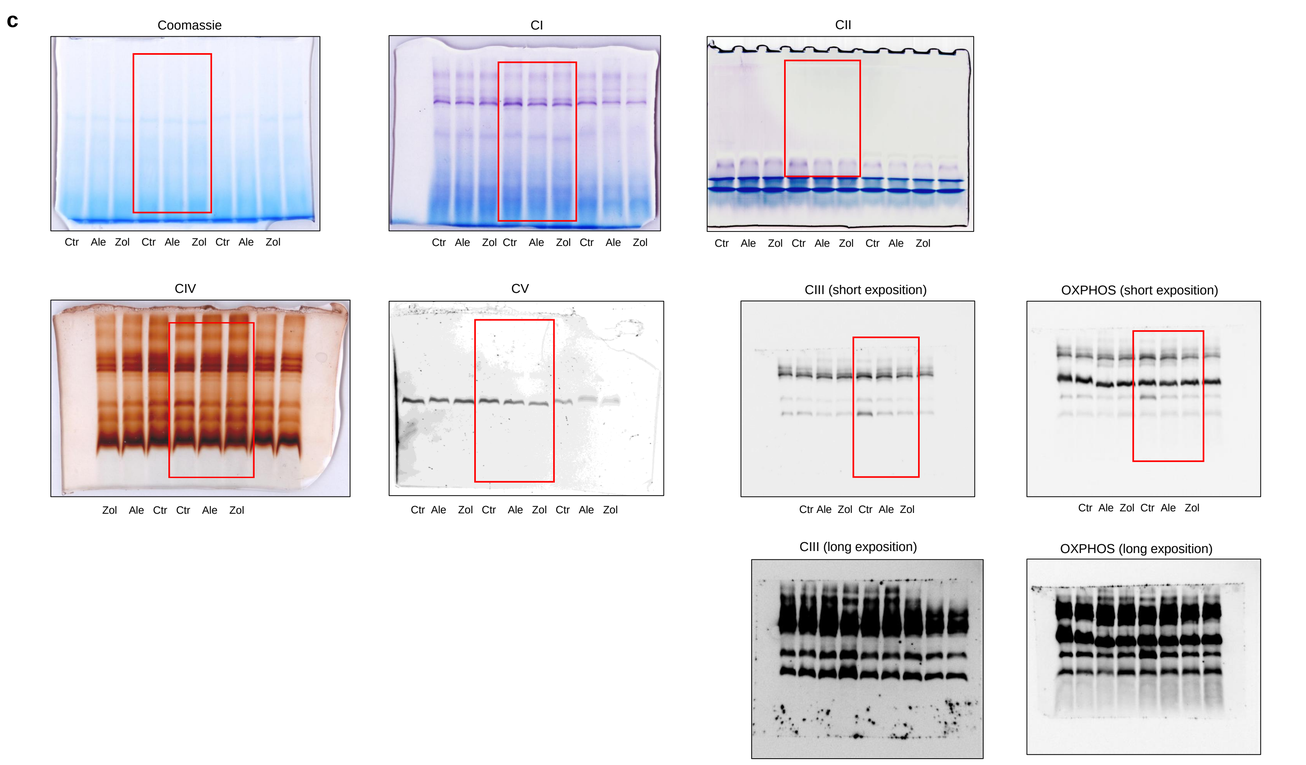


Images with visible membrane edges

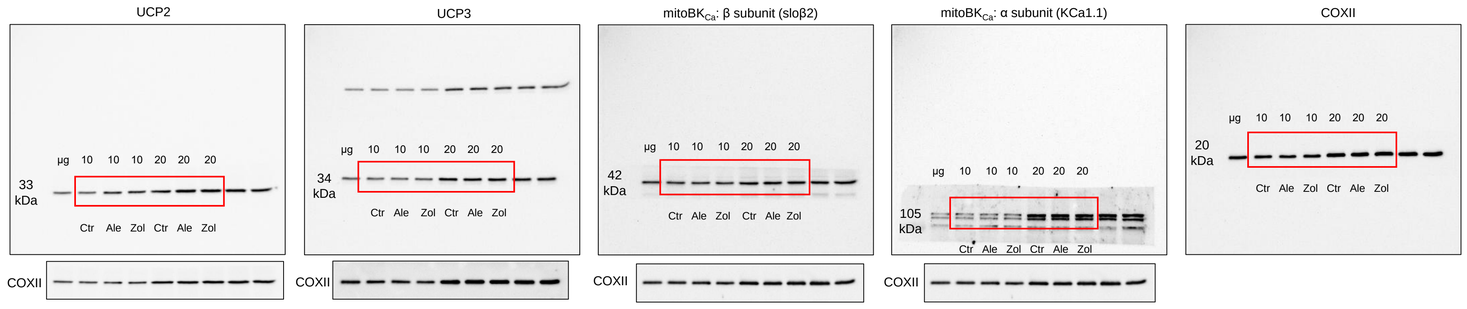


Composite images: chemiluminescence + nitrocellulose membrane





Supplementary Fig. S3. Images used to prepare Fig. 5. **a,** Unprocessed images of blots shown in Fig. 5a. **b,** Images used to perform the calculations shown in Fig. 5a. **c,** Uncropped images of gels and blots presented in Fig. 5b. Ctr, control mitochondria; Ale, mitochondria of alendronate-treated cells; Zol, mitochondria of zoledronate-treated cells.



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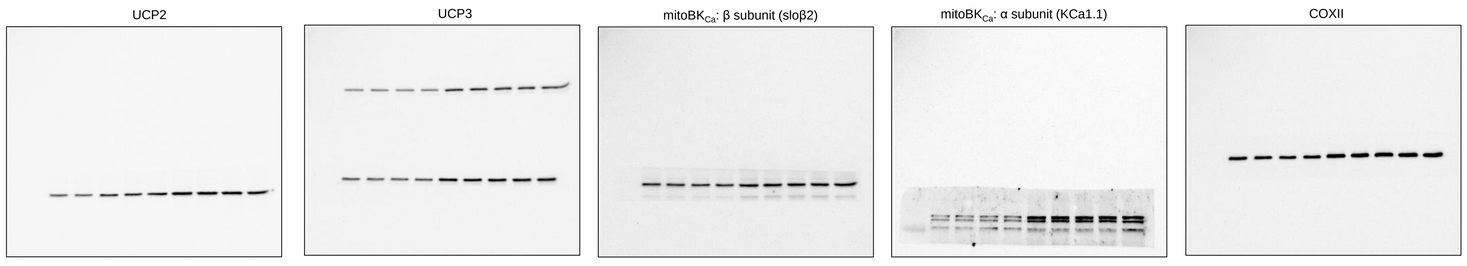
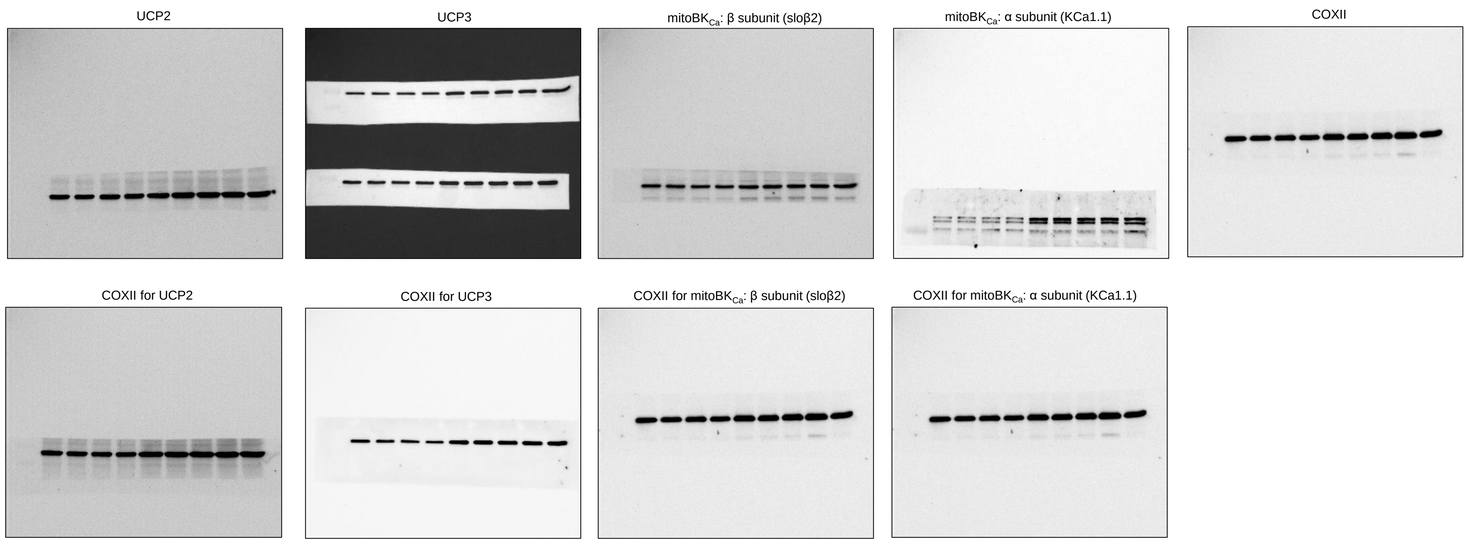
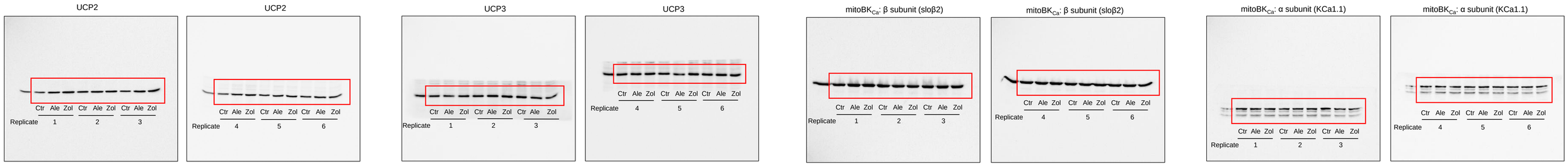


Images with visible membrane edges or composite images: chemiluminescence + nitrocellulose membrane



Supplementary Fig. S4. Uncropped images of blots shown in Fig. 6a. Ctr, control mitochondria; Ale, mitochondria of alendronate-treated cells; Zol, mitochondria of zoledronate-treated cells.



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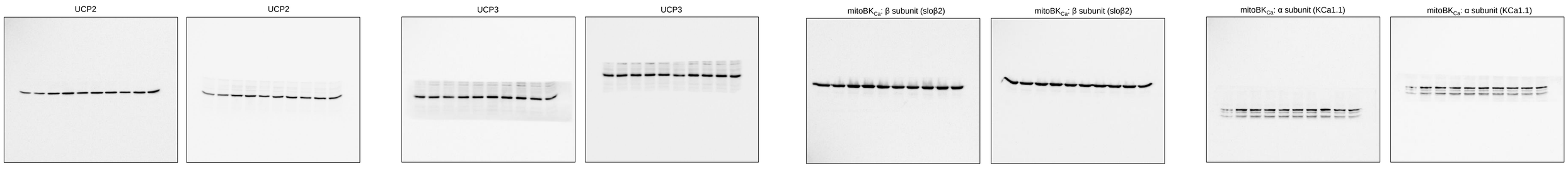
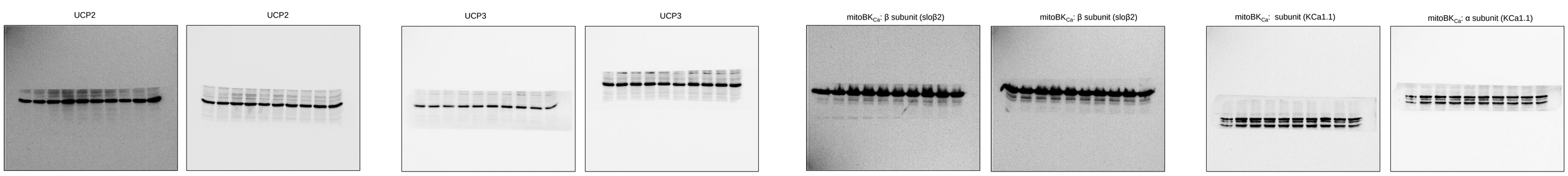
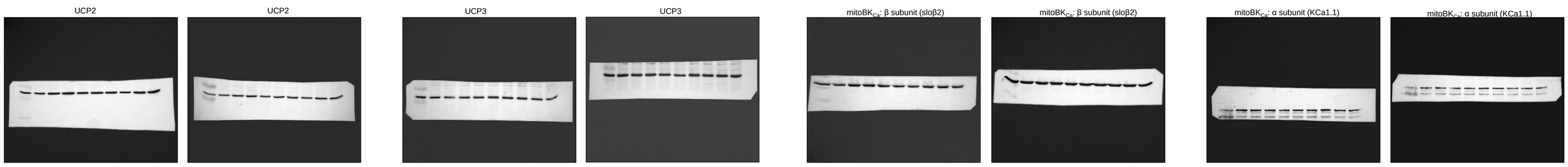


Images with visible membrane edges



Composite images: chemiluminescence + nitrocellulose membrane



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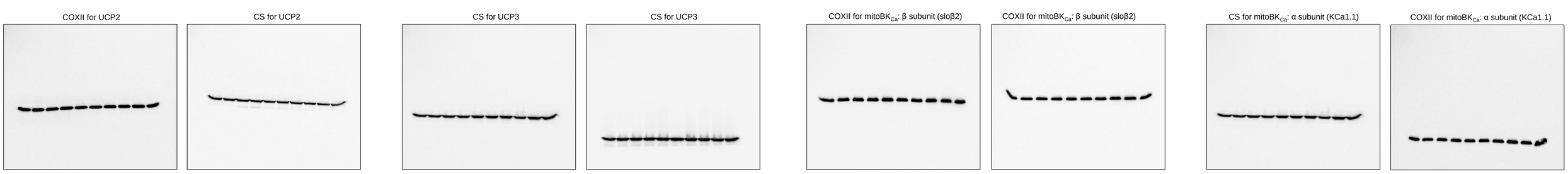

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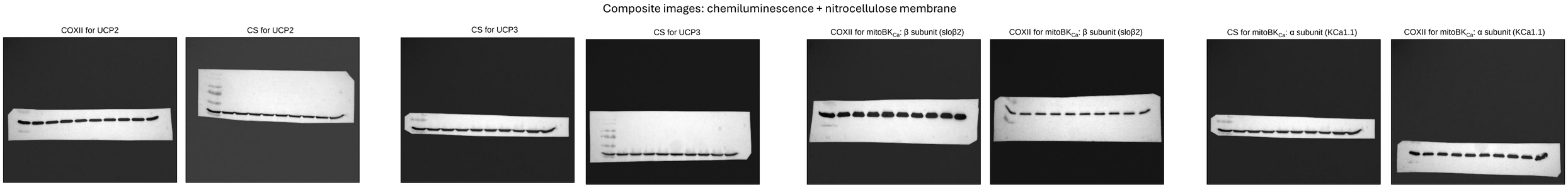

Supplementary Fig. S5. Images used for densitometric analysis shown in Fig. 6a. Ctr, control mitochondria; Ale, mitochondria of alendronate-treated cells; Zol, mitochondria of zoledronate-treated cells.

Review

Consequences of Inhibition of the Mevalonate Pathway by Bisphosphonates at the Cellular and Mitochondrial Levels

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Keywords: mevalonate pathway; mitochondria; nitrogen-containing bisphosphonates

Review

Consequences of Inhibition of the Mevalonate Pathway by Bisphosphonates at the Cellular and Mitochondrial Levels

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Abstract

Nitrogen-containing bisphosphonates (N-BPs) are commonly used drugs in the treatment of bone diseases due to their potent inhibition of the mevalonate pathway, leading to disrupted protein prenylation and reduced osteoclast activity. Although N-BPs are effective in reducing bone resorption, increasing evidence indicates their side effects on various non-skeletal cells. At the cellular level, N-BPs may reduce viability, modulate inflammatory responses, trigger apoptosis, disrupt cytoskeletal organization and influence signaling and energy metabolism. N-BPs may also impair prenylation of proteins essential for mitochondrial dynamics and quality control, and may disrupt Ca^{2+} homeostasis. By inhibiting the mevalonate pathway, N-BPs may lead to the reduction of key components of the mitochondrial respiratory chain, such as coenzyme Q (CoQ) and *a*-heme. These effects can contribute to impaired mitochondrial respiratory function, increased oxidative stress and mitochondria-dependent apoptosis, effecting cellular energy metabolism and viability. These findings underscore the multifaceted impact of N-BPs beyond bone, emphasizing the importance of mitochondrial health and energy metabolism in understanding their broader biological effects and potential adverse outcomes.

Keywords: mevalonate pathway; mitochondria; nitrogen-containing bisphosphonates

1. Introduction

1.1. History of Bisphosphonates

Osteoporosis is the most common metabolic bone disease, particularly prevalent among postmenopausal women. As the world's population ages, the incidence of osteoporosis is increasing, with over 200 million people now suffering from the disease [1]. Bone health is maintained by a delicate balance between bone resorption by osteoclasts and bone formation by osteoblasts. This balance can be disturbed by many factors, including aging, genetic disease, lifestyle and endocrine disorders [2]. The primary goal of osteoporosis treatment is the prevention of fractures, which can be achieved either by stimulating bone formation or, more commonly, by inhibiting bone resorption. Bisphosphonates (BPs) are the most commonly prescribed antiresorptive drugs, approved as first-line treatment for osteoporosis, Paget's disease and bone diseases in cancer patients [3]. Their clinical use has also been extended to the treatment of bone metastases and other diseases associated with increased bone turnover.

BPs are stable synthetic pyrophosphate (PPi) analogues, in which the central carbon atom replaces the PPi oxygen bridge and carries two variable side chains, R1 and R2 (Figure 1). Historically, before their potential in preventing bone resorption was discovered in 1969 [4,5], they were first used industrially for their calcium-chelating properties [6,7] as water softeners, corrosion inhibitors and

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fertilizers. Manipulation of BP side chains has led to the discovery of new compounds with different potencies, while the synthesis of BPs containing a nitrogen atom in the R2 alkyl chain has created a new type of drugs with significantly improved antiresorptive properties [8].

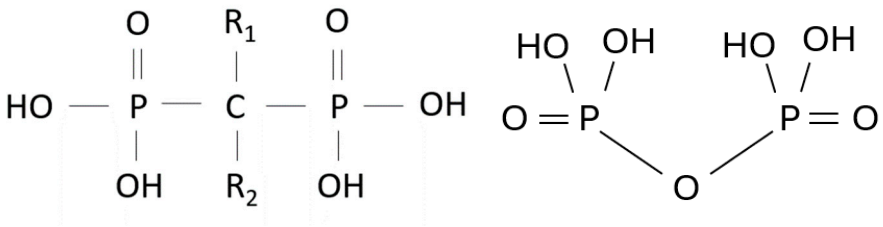

Figure 1. General structure of pyrophosphate and bisphosphonate.

1.2. Types of BPs

BPs are divided into two main groups based on their R2 side chain – non-nitrogen-containing bisphosphonates and nitrogen-containing bisphosphonates (N-BPs) [9]. N-BPs can be further divided into second-generation (including alendronate) and more efficient third-generation (including ibandronate and zoledronate) drugs. First-generation BPs – non-nitrogen-containing compounds such as etidronate and clodronate – can interfere with ATP-dependent enzymes by generating non-hydrolyzable ATP analogues [10]. These analogues compete with ATP, ultimately inducing apoptosis in osteoclasts. Most often, these types of BPs contain a simple hydroxyl group in the R1 chain, while the R2 chain may contain a chlorine atom (in clodronate) or an alkyl group (in etidronate). Clodronate is metabolized by class II aminoacyl-tRNA synthases to adenosine-5′-(β,γ-dichloromethylene) triphosphate ($AppCCl_2p$), a toxic ATP analogue that accumulates and disrupts cellular energetics and other cellular processes [11,12]. Non-hydrolyzable ATP analogues may reduce cytokine release, offering potential in treating inflammatory conditions. They reach mitochondria, changing oxygen consumption and depolarizing the mitochondrial membrane potential [13].

In contrast, N-BPs act by inhibiting farnesyl diphosphate synthase (FPP synthase) in the mevalonate pathway, preventing prenylation of proteins essential for osteoclast survival and function (e.g., membrane ruffling and stress fibre assembly) [13–15]. Greater efficacy of N-BPs compared to first-generation BPs is attributed to their greater affinity for bone mineral [14]. Bone-targeting properties of N-BPs provide selective accumulation at resorption sites, where they effectively inhibit osteoclast recruitment, activity and induce apoptosis. The most commonly used N-BPs are alendronate, prescribed primarily to prevent bone fractures, and zoledronate, a more potent agent used to prevent pathological fractures or treat bone loss associated with cancer [15].

1.3. Side Effects of N-BPs

N-BPs can be administered intravenously or orally. However, the oral administration is linked with poor absorption and has many contradictions. The negative charge of N-BPs impedes their transport across the lipophilic membrane. A similar effect is achieved by complexing with calcium ions, to which they owe their high affinity to sites of intense bone resorption. Most of the contradictions of N-BPs use are connected to the frequent comorbidities of osteoporosis, e.g., inflammatory bowel diseases (Crohn's disease, ulcerative colitis, celiac disease), small bowel resection or gastojejunostomies [16]. Intravenous treatment with N-BPs, particularly zoledronate, shows greater drug adhesion to the bone surface, with approximately 50–70% of the dose binding to bone minerals within 6–10 hours. Compared to the low oral bioavailability (~5%), intravenous treatment demonstrates its superiority [17,18].

Use of alendronate, a second-generation bisphosphonate, has tripled from 2.7% in the 1990s to 7.8% in less than a decade [19]. The global market of N-BPs is expected to be 9.8 billion dollars by 2032 [20]. Given this, the side effects of these medications have been extensively studied (Table 1).

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Upper gastrointestinal side effects are most commonly reported with oral N-BPs. Failure to maintain an upright position for 60 minutes after taking the drug caused esophagitis in patients [21].

Table 1. Documented major side effects of BP therapy [22].

Side effects	BPs
Osteomalacia	Etidronate
Hypocalcemia	Zoledronate, pamidronate, clodronate
Acute phase reaction	Zoledronate, ibandronate
Gastrointestinal side effects	Orally administered N-BPs, alendronate
Nephrotoxicity, renal failure	Zoledronate, pamidronate, ibandronate, alendronate
Ocular side effects	Pamidronate, zoledronate
Bisphosphonate-related osteonecrosis of the jaw (BRONJ)	Zoledronate

The most feared side effect of intravenous N-BPs is osteonecrosis of the jaw, which is likely due to the antiangiogenic properties of N-BPs. Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is thought to be related to the use of high doses of the drugs, but the condition also affects patients taking lower doses. More recently, BRONJ has been proposed to be caused by the presence of Actinomyces infection, decreased bone turnover, inhibition of angiogenesis and immune dysregulation [23]. Ziebart et al. suggested that the effect of N-BPs on the onset of BRONJ could be reversed [24]. By studying the products of the mevalonate pathway, they found that one of them, geranylgeraniol (GGOH), when added to in vitro cultures of HUVEC cells, reversed the effects of clodronate, zoledronate, pamidronate and ibandronate.

2. Mevalonate Pathway – A Biological Role and Products

The mevalonate pathway is a key metabolic pathway that supplies isoprenoid units and plays a central role in the biosynthesis of important molecules, including cholesterol, steroid hormones, heme *a* and coenzyme Q (CoQ) (Figure 2) [26,27]. The mevalonate pathway has been the subject of in-depth research due to its importance in the pathophysiological processes of cancer, cardiovascular and neurodegenerative diseases [27].

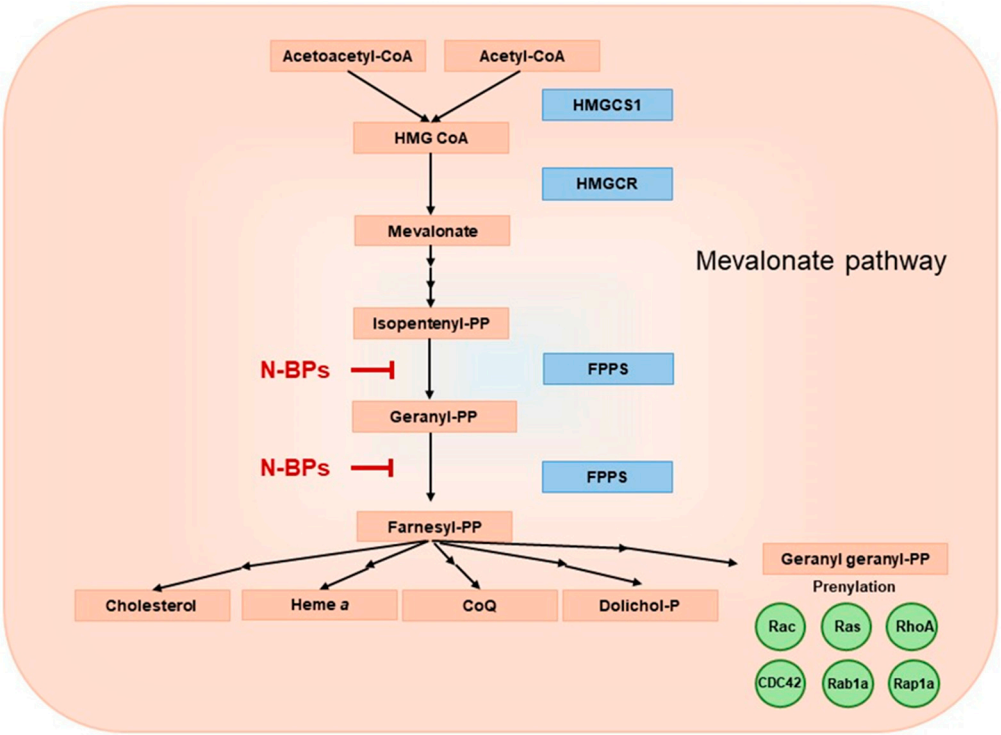

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Figure 2. Mevalonate pathway diagram. HMGCS1 – hydroxymethylglutaryl-CoA synthase, HMG CoA – 3-hydroxy-3-methylglutaryl coenzyme A, HMGCR – 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, FPPS – farnesyldiphosphate synthase.

2.1. Cholesterol

Cholesterol, the major sterol in mammals, is found in all cell membranes and forms lipid rafts that are an important part of signaling complexes. It constitutes about one third of lipids in the plasma membrane, packaging phospholipids into membranes and insuring their fluidity and barrier function [28]. The sterols present in cell membranes are mainly produced endogenously. If cholesterol production is upregulated, sterol regulatory element-binding proteins (SREBP) step in, step in, regulating the expression of genes involved in lipid synthesis and maintaining a narrow, nontoxic range of this molecule [29]. Cholesterol is also essential as a precursor for the production of steroid hormones. Increasing evidence suggests that cancer cells are highly dependent on high cholesterol levels, moderating the antiviral response of type I interferon (IFN) in macrophages while avoiding detection by the immune system [30].

2.2. Dolichol

Dolichol, a polyprenol, is an another product of the mevalonate pathway which plays an important role in protein glycosylation. As an essential oligosaccharide carrier, it is involved in the synthesis of C-mannosylation, glycosylphosphatidylinositol (GPI)-anchored protein and N-glycans [31]. It has been described over 30 years ago that the level of dolichol increases with age in the human brain. Although in neurodegenerative diseases, such as Alzheimer's, the situation is opposite showing possible increases in dolichyl phosphate glycosylation as the part of the pathophysiological processes of this disease [32].

2.3. CoQ

CoQ is a lipid-soluble molecule found in cell membranes. Although the human body is able to synthesize it, it is also abundant in our diet. CoQ is an important electron transport carrier of the respiratory chain involved in producing ATP by mitochondrial oxidative phosphorylation system (Figure 3) [33]. CoQ moves across the inner mitochondrial membrane, transporting electrons from substrate dehydrogenases (complexes I and II), where it is reduced, to complex III, facilitating electron transport in the respiratory chain. In addition, CoQ is an antioxidant and can replenish the pool of other antioxidants, such as vitamin C or E, by reducing their oxidized form. The antioxidant properties of CoQ are largely dependent on processes that renew the reduced pool of $CoQH_2$ [34]. However, if the molecule is not fully reduced, a semiquinone radical can be formed, which reacts with oxygen to produce a precursor of various reactive oxygen species (ROS) – the superoxide anion. CoQ levels naturally decline with age, and decreased concentrations have also been observed in various pathological conditions, including diabetes, cancer and neurodegenerative disorders [35].

2.4. Heme a

Heme *a* is an essential molecule in organisms that depend on aerobic respiration. It is embedded in electron transport chains as a prosthetic group in respiratory oxidases containing cytochrome *a* [36]. In mammals, hemes $a + a_3$ are components of cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) (Figure 3). Mutations in the COX15 gene encoding heme *a* synthase cause fatal infantile hypertrophic cardiomyopathy [36]. On the other hand, increased levels of heme *a* were found in Alzheimer's disease patients [37]. Furthermore, the accumulation of Cox-1 protein and other mitochondrial markers in autophagocytic vesicles is associated with a higher level of mitochondrial turnover.

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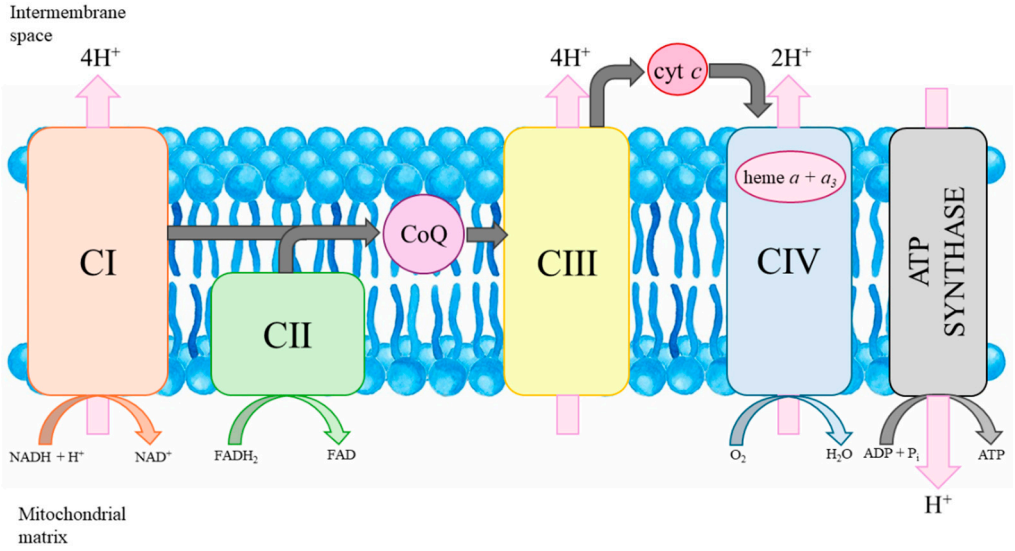

Figure 3. Mitochondrial oxidative phosphorylation system. cyt *c*, cytochrome *c*; CI-CIV, respiratory chain complexes.

2.5. Protein Prenylation

Protein prenylation is essential for cell growth, cell division, receptor trafficking and cell polarization. It is the first step in targeting and binding to the cell membrane and also mediates protein-protein interactions. Interest in the process of protein prenylation increased following the description of protein prenylation as being required for the malignant activity of oncogenic Ras proteins [38].

Two main isoprenoid molecules involved in protein prenylation, which are produced in mevalonate pathway, are farnesyl-diphosphate (FPP) and geranylgeranyldiphosphate (GGPP) [39]. GGPP is crucial for membrane localization of small GTP-binding proteins such as Ras, Rho, Rac and Rap. Ras and Rho GTPases require post-translational modifications such as farnesylation or geranylgeranylation. Prenylation allows them to associate with lipid membranes and act as a molecular switch that toggles between an inactive GDP-bound state and an active GTP-bound state. N-BPs inhibit the post-translational modifications of small GTPases by inhibiting the FPP synthase [39]. The concomitant use of N-BPs and statins, other mevalonate pathway inhibitors that block 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoA reductase), has been proposed as an effective strategy for the treatment of malignant tumors due to the inhibition of FPP and GGPP biosynthesis from two points in the pathway [40].

To inhibit tumor growth and induce apoptosis of cancer cells, a new treatment method was proposed in which zoledronate was placed in a liposome, which allowed to bypass the high affinity of the drug for bone [41]. Interestingly, this technique led to the inhibition of FPP synthase activity, loss of protein prenylation, and also allowed zoledronate to cross the blood-brain barrier, as detected in the brains of the tested mice. It has been suggested that prenylation inhibitors should be further investigated as a potential new class of anti-cancer drugs.

3. Inhibition of the Mevalonate Pathway by N-BPs

3.1. Effects on the Cellular Level

3.1.1. Effects on Bone Cells

The main target of N-BPs are osteoclasts. Selective absorption of N-BP to sites with high bone resorption rates causes the drugs to come into close contact with osteoclasts, which translates into their high safety and efficacy [42]. N-BPs reduce bone resorption by inhibiting osteoclast recruitment,

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resorption activity and inducing apoptosis. N-BPs, by inhibiting FPP synthase, prevent protein prenylation, which is crucial for obtaining resorption activity by osteoclasts. The correlation between FPP synthase inhibition and antiresorptive activity of N-BPs was shown over 20 years ago by Dunford et al. [43].

Despite the expected effect of reducing the rate of bone resorption, N-BPs also act on other cells with which they come into contact [44]. It has been shown that these drugs can alter the expression of osteoprotegerin (OPG) and macrophage colony stimulating factor (M-CSF) in osteoblasts, affecting osteoblastogenesis. Variable effects on the proliferation and differentiation of osteogenic cells have been observed when different concentrations of N-BPs were administered. The anti-apoptotic properties of N-BPs towards osteoblasts are observed when using concentrations ranging from 10^{-6} to 10^{-8} M. The pro-apoptotic events in osteoblasts and osteocytes were observed after increasing the dose to 10^{-4}-10^{-5}M.

N-BPs inhibit bone resorption leading to lower serum calcium levels [15,45,46]. The P-C-P moiety in N-BPs is responsible for quick binding to the bone mineral. However, the different substitutions in the R_1 and R_2 positions in the carbon atom allow them to chelate Ca^{2+} more effectively [47]. Most studies focus on N-BPs effects within osteoclasts or their precursors, particularly concerning calcium-related ion channels. For example, Sheng-Nan et al. investigated the effects of ibandronate on intermediate-conductance Ca^{2+}-activated K^+ (IK_{Ca}) channels in the osteoclast precursor cell line RAW 264.7 [48]. They found that ibandronate inhibited IK_{Ca} channel activity, resulting in membrane depolarization and reduced cell migration.

3.1.2. N-BP Effects on off-Target Cells

The way in which BP treatment alters cellular processes and leads to apoptosis depends on their molecular structure [10]. Non-nitrogen containing BPs bind to ATP, leading to the production of non-hydrolyzable ATP analogues that compete with the nucleotide, resulting in impaired energy metabolism and apoptosis. N-BPs inhibit FPP synthase, a key enzyme of the mevalonate pathway, thereby preventing the prenylation and activation of small GTPases that are essential for cytoskeletal organization, vesicular trafficking, cell survival and other cellular processes. In vitro studies demonstrating the toxicity of both types of BPs (N-BPs and non-nitrogen containing BPs) do not contain information allowing comparison of the concentrations of these drugs with the concentrations observed in the plasma of patients undergoing BP therapy. In the case of studies with N-BPs, depending on the cell line, their concentrations range from 1 μM in the human endothelial cell line (EA.hy926), 5 μM in the human epithelial cell line (HaCaT), to even 100 μM in the umbilical vein endothelial cell line (HUVEC) [49–51].

Anti-Cancer Effects, Cytoskeleton Alterations

Relatively little is known about the effects of N-BPs on tissues other than bone. Most of the existing knowledge comes from studies of cancer cells, as N-BPs are used in anti-cancer therapies [52]. N-BPs, such as zoledronic acid, are widely used to prevent skeletal-related events in cancer patients with bone metastases. Beyond their bone-targeting properties, preclinical studies have demonstrated that N-BPs exert direct anti-cancer effects. These effects include the inhibition of tumor cell proliferation, induction of apoptosis, and modulation of the tumor microenvironment. For instance, N-BPs have been shown to stimulate the expansion and cytotoxic activity of human T cells, which possess potent anti-tumor capabilities. The anti-tumor effect of N-BPs by blocking the prenylation of small GTPases has been explained by the disruption of cytoskeletal organization [53]. After adding 10^{-5} M alendronate to human prostate cancer (PC-3) cells, Virtanen at al. observed significant changes in the organization of F-actin and cofilin level. Phosphorylation of cofilin is crucial for actin filament elongation, which is carried out by Rho GTPases. This observation led to the hypothesis that N-BPs, which cause loss of prenylation of small GTPases, may be associated with lower levels of phosphophilin, which disrupts the balance between phosphorylated and unphosphorylated cofilin, affecting actin dynamics [54,55]. Morphological changes caused by

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disturbances in the polymerization of the actin cytoskeleton were observed during zoledronate treatment in fibroblasts, vascular smooth muscle cells and tumor cells [56–58].

Effects Related to Nephrotoxicity

In human embryonic kidney (HEK-293) cells, zoledronic acid increase ROS production and induce oxidative damage, as evidenced by altered expression of genes related to oxidative stress and apoptosis [59]. Additionally, endoplasmic reticulum (ER) stress-mediated apoptosis has been observed. Studies in zoledronate-treated rats and renal tubular HK-2 cells suggest that zoledronate-induced nephrotoxicity is linked to disruptions in glutathione biosynthesis and the tricarboxylic acid (TCA) cycle, leading to excessive ROS production, oxidative stress, and inflammation, thereby leading to nephrotoxicity [60].

Effects on Endothelial Cell Viability, Inflammation and Apoptosis

N-BPs have been shown in vitro to reduce the viability of human endothelial cells, induce their inflammatory response and impair the endothelial differentiation potential of human mesenchymal stem cells [51,61,62]. One of the signaling pathways involved in endothelial cell proliferation, apoptosis and angiogenesis is the EGFR/Akt/PI3K pathway, which begins with activation of the epidermal growth factor receptor (EGFR) by ligands, which then triggers activation of phosphatidylinositol 3-kinase (PI3K) and Akt, releasing the Bcl2-related cell death antagonist [63]. The Akt pathway is responsible for the activation of nuclear factor κB (NFκB), reprograming the expression of genes whose products are involved in stress responses, inflammation and inhibition of apoptosis. N-BPs are considered good candidates for anti-cancer activity because they inhibit the key cell survival pathway by inhibiting the activation of Akt and NFκB [64]. Moreover, zoledronate has been shown to impair endothelial cell function and survival by inhibiting the extracellular signal regulated protein kinase 1/2 (ERK1/2) pathway, other prenylation-dependent signaling cascade [65]. Importantly, ERK1/2 also serves as an anti-inflammatory signal to prevent inflammatory signaling in endothelial cells [66]. Endothelial cells treated with zoledronate but not alendronate exhibited a significant decrease in active phospho-ERK1/2 levels, along with increased expression of inflammatory markers [intercellular adhesion molecule 1 (ICAM1) and cytokine interleukin-6 (IL6)], indicating endothelial cell activation and the initiation of local inflammation through the upregulation of inflammatory cytokines and adhesion molecules [51]. In osteoblasts and fibroblasts, zoledronate, unlike alendronate, also induced an increased level of inflammatory cytokines [67]. These observations indicate that zoledronate induces a more potent inflammatory response than alendronate, although both N-BPs have a similar effect on ERK1/2 phosphorylation [51].

Effects on Endothelial Cell CoQ Homeostasis and Energy Metabolism

N-BPs, by inhibiting the mevalonate pathway, lead to the inhibition of synthesis of CoQ, an important intracellular antioxidant that is present in all cell membrane [68–70]. Reduced CoQ ($CoQH_2$), by binding free radicals, inhibits lipid peroxidation processes and prevents oxidative modifications of DNA and proteins. Decreased CoQ levels due to age or blockade of the mevalonate pathway by drugs (e.g., statins) may result in oxidative stress and damage. For example, patients with coronary heart disease have lower levels of plasma CoQ, which contributes to lipid peroxidation and DNA damage [71]. In 2014, for the first time (and for a long time the only) an association was observed between N-BP therapy and reduced levels of CoQ10 in the plasma of postmenopausal women [72]. However, to date, CoQ10 supplementation is rarely recommended [72,73]. Reductions in CoQ levels in tissues or organs during N-BP treatment have not yet been investigated, apart from our findings in endothelial cells [51,73]. We recently showed that both alendronate and zoledronate significantly reduced CoQ levels in EA.hy926 human endothelial cells [51]. In particular, in vitro treatment with 2.5 μM zoledronate caused a 60% decrease in cellular CoQ content. N-BPs disrupted the cellular CoQ redox homeostasis by decreasing the CoQ redox state ($CoQH_2$/CoQ). The significant

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N-BP-induced CoQ deficiency seems to contribute to metabolic reprogramming, including reduced mitochondrial respiratory function and reduced ATP production. A general decline in mitochondrial respiration has been observed in N-BP-treated endothelial cells, particularly with stronger reducing substrates such as pyruvate and glutamate. However, the oxidative capacity as well as aerobic metabolism in N-BP-treated endothelial cells were increased. Impairment of the oxidative phosphorylation system in N-BP-treated endothelial cells was evidenced by reduced ATP-linked respiration with stronger reducing substrates and decreased cellular ATP levels. These alterations in oxidative metabolism resulted in elevated intracellular oxygen levels. N-BP-treated endothelial cells showed significant increases in histone lysine-specific demethylase 6A (KDM6A), a direct oxygen sensor involved in the regulation of gene transcription, along with significant reductions in hypoxia-inducible factor 1α (HIF1α), a key marker of cellular hypoxia. These observations suggest the presence of elevated oxygen levels in N-BP-treated cells, which may enhance reactive oxygen species (ROS) production and contribute to oxidative stress. In N-BP-treated endothelial cells, a decrease in CoQ level, particularly its antioxidant-active reduced form, was accompanied by increased total and mitochondrial ROS production, resulting in oxidative stress, as evidenced by elevated levels of the antioxidant enzymes glutathione reductase and superoxide dismutase (SOD1). These studies represent the first comprehensive investigation into the effects of N-BPs on cellular energy metabolism, using endothelial cells as a model system.

Effects on Lipid Metabolism

In vivo, zoledronate treatment reduced hepatic lipid accumulation in mice fed a high-fat diet, suggesting a potential regulatory effect on lipid metabolism in liver cells [74]. Additionally, zoledronate increased the expression of genes involved in fatty acid oxidation and reduced the size of lipid droplets in hepatocytes of treated mice. An increase in palmitate oxidation was observed in EA.hy926 endothelial cells treated with zoledronate or alendronate, indicating a metabolic shift towards fatty acid catabolism [51]. In contrast to these findings, Cheng et al. showed that zoledronate induces fatty acid accumulation in renal HK-2 cells [75]. It has been proposed that, although β-oxidation of fatty acids is the primary energy source for renal tubular epithelial cells, excessive fatty acid accumulation may contribute to nephrotoxicity and the development of tissue fibrosis or renal disease.

Effects on Authophagy, Mitochondrial Dynamics and Turnover

Defective autophagy has also been linked to a severe reduction in protein prenylation, which may lead to inflammasome activation and subsequent cell death [76]. However, the effects of NBPs, as inhibitors of the mevalonate pathway, on autophagy in non-bone cells remain largely unexplored.

The mevalonate pathway supports the prenylation of small GTPases, including Rab proteins, which are essential for mitochondrial quality control processes such as fission and mitophagy [76–78]. However, this aspect remains poorly investigated in the context of N-BP inhibition of the pathway and so far only one study shows that N-BPs can affect mitochondrial dynamics and turnover [51]. In EA.hy926 human endothelial cells treated with N-BPs, no changes were observed in the levels of mitochondrial biogenesis markers, including peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) and nuclear factor erythroid 2–related factor 2 (NRF2) [51]. However, the reduction in mitochondrial fission markers – mitochondrial fission factor (MFF) and active phospho-dynamin-related protein 1 (phospho-DRP1) – along with unchanged levels of the fusion marker optic atrophy protein 1 (OPA1), suggests reduced mitochondrial clearance via mitophagy in N-BP-treated endothelial cells, likely as a consequence of ERK1/2 signaling downregulation. These studies provide the first evidence that N-BPs alter mitochondrial dynamics and turnover.

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Effects on Calcium Homeostasis

N-BPs can lower plasma calcium levels by inhibiting bone resorption, thereby reducing the release of calcium into the bloodstream [15,45,46]. Currently, there is limited evidence that N-BPs disrupt calcium homeostasis in tissues beyond bone and blood. For example alendronate has been shown to affect cellular calcium dynamics in cardiomyocytes in vitro [79].

3.2. N-BP Effects on the Mitochondrial Level

Mitochondria are the site of oxidative phosphorylation, the process by which cells produce ATP from nutrients [80,81]. Oxidative phosphorylation occurs across the inner mitochondrial membrane via the electron transport chain and ATP synthase (Figure 3). Mitochondria are also key guardians of cellular homeostasis. In addition to synthesizing ATP via oxidative phosphorylation, mitochondria are also major sources of ROS. While ROS at the physiological level function as critical signaling molecules involved in maintaining cellular homeostasis, excessive ROS production becomes detrimental. When the balance is disturbed, elevated levels of ROS can damage lipids, proteins, and DNA, contributing to oxidative stress and playing a central role in the pathogenesis of various diseases, including neurodegeneration, cancer, and cardiovascular disorders. In addition, impaired mitochondria release apoptotic factors that act as signals that induce cell death. Mitochondria also play an important role in Ca^{2+} homeostasis by buffering intracellular calcium levels, thereby supporting proper cell function and survival [82].

Effects on Mitochondria-Induced Apoptosis

Mitrofan et al. investigated the mechanism of zoledronate-induced apoptosis in the HF28RA human follicular lymphoma cells and identified key mitochondrial events involved in this process [83]. Zoledronate treatment led to mitochondrial membrane permeabilization, which resulted in the release of cytochrome *c* into the cytosol, activation of caspase-3, and DNA fragmentation, i.e., hallmarks of mitochondrial-dependent apoptosis. The study identified inhibition of the mevalonate pathway as a major trigger of this response. Geranylgeraniol (GGOH) supplementation, which restores prenylation of proteins downstream of the blocked pathway, effectively rescued cells from zoledronate-induced apoptosis, underscoring the importance of prenylated proteins in maintaining mitochondrial integrity and cell survival. Thus, N-BPs can activate the intrinsic pathway of apoptosis by interfering with mitochondrial function through loss of membrane potential, oxidative stress, and impaired protein prenylation. Although the exact molecular link between N-BP and mitochondrial apoptosis signaling remains unclear, the results support potential therapeutic strategies combining N-BPs with agents that modulate mitochondrial sensitivity or protein prenylation to enhance their efficacy, particularly in resistant cancer cells.

Effects on Mitochondrial Calcium Homeostasis

Some evidence suggests that N-BPs may influence mitochondrial calcium handling in kidney cells. For instance, studies have reported reduced calcium release from renal mitochondria in vitro [84] as well as increased mitochondrial calcium levels in vivo [85]. Interestingly, etidronate has been shown to protect the kidney from ischemic injury [86]. This protective effect is proposed to result from enhanced mitochondrial calcium sequestration, which helps prevent intracellular Ca^{2+} overload.

N-BP-Induced Mitochondrial Heme a Decrease

Mevalonic acid serves as a precursor for the synthesis of isoprenoid units-containing heme *a*, an essential prosthetic group of cytochrome *c* oxidase (complex IV) [87]. In EA.hy926 endothelial cells, treatment with inhibitors of the mevalonate pathway, zoledronate or alendronate, led to a reduction in cytochromes $a + a_3$, suggesting a reduction in the heme *a* content of complex IV [73]. Despite this reduction, the maximum enzymatic activity of complex IV in intact endothelial mitochondria remained unchanged compared with control untreated cells. Interestingly, this is the only study to

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date to directly examine the effect of N-BPs on mitochondrial heme *a* levels, emphasizing the need for further studies to elucidate the broader implications of N-BP-induced disruption of mitochondrial heme *a* biosynthesis and respiratory chain integrity.

N-BP-Induced Changes in Mitochondrial Respiratory Function

Mitochondrial dysfunction induced by N-BPs remains an underexplored area and relatively few studies have addressed this aspect. In mitochondria isolated from the kidneys of zoledronate-treated rats, significant decreases in mitochondrial dehydrogenase activities, mitochondrial membrane depolarization and permeabilization as well as ATP depletion were observed [88]. In mitochondria isolated from N-BP-treated endothelial cells, an overall reduction in the oxidation of respiratory substrates was observed, with the exception of increased fatty acid oxidation [73]. These mitochondria exhibited reduced respiratory rates, diminished membrane potential and lower efficiency of ATP synthesis during the oxidation of complex I and II substrates. Moreover, in the mitochondria of endothelial cells treated with N-BPs, a reorganization of respiratory chain supercomplexes was observed, notably a downregulation of the complex III_2 + IV supercomplex and III_2 dimer . This changes were accompanied by reduced protein levels and enzymatic activities of complexes II, III, and V, indicating impaired structural and functional integrity of the electron transport chain.

N-BP-Induced mtCoQ Deficiency

N-BPs as inhibitors of the mevalonate pathway may disturb the synthesis of isoprenoid-derived molecules, including mitochondrial CoQ (mtCoQ), a key electron carrier in the mitochondrial electron transport chain (Figure 3) [69,70]. Reduced mtCoQ ($CoQH_2$) participates in the production of mitochondrial ROS through the formation of superoxide/H_2O_2 due to electron leakage from the semiubiquinone radical at the mtCoQ-biding sites of the respiratory chain. mtCoQ redox homeostasis, maintained through its cycling between oxidized and reduced forms, is crucial for efficient electron transport and the regulation of oxidative stress [69].

Recently, we demonstrated for the first time that treatment with N-BPs leads to a significant reduction in mtCoQ level. In EA.hy926 endothelial cells treated with alendronate or zoledronate, a 45–55% reduction in total mtCoQ was observed [73]. Importantly, the pool of reduced mtCoQ ($mtCoQH_2$), which is essential for its antioxidant function, was lost. The disrupted mtCoQ redox homeostasis induced a compensatory response characterized by increased expression of mitochondrial antioxidant proteins, including superoxide dismutase 2 (SOD2) and uncoupling protein 2 (UCP2). Moreover, N-BP treatment resulted in a significantly increase in mitochondrial H_2O_2 production, which has been attributed to increased mtCoQ reduction level (elevated $mtCoQH_2$/mtCoQtot ratio). These findings suggest that decreased mitochondrial CoQ levels, due to mevalonate pathway inhibition by N-BPs, may impair mitochondrial respiratory function and disrupt CoQ redox balance, leading to elevated mitochondrial ROS production. These results underscore the need for further studies on mtCoQ deficiency induced by N-BP treatment. Severe mtCoQ depletion, coupled with excessive mitochondrial ROS production, can disrupt mitochondrial integrity and cellular homeostasis. Such dysfunctions are known to contribute to the pathogenesis of several diseases, including cardiovascular and neurodegenerative diseases, and may be associated with accelerated aging and reduced lifespan [89]. Elucidation of the mechanisms underlying mtCoQ depletion and its downstream effects may point to new strategies to alleviate N-BP-induced mitochondrial toxicity, particularly through CoQ10 supplementation, which may help restore mitochondrial function and redox balance.

4. Conclusions and Future Perspectives

Although widely used N-BPs are clinically effective in treating bone resorption disorders, they are increasingly recognized as drugs with a broad spectrum of adverse effects resulting from

inhibition of the mevalonate pathway in non-skeletal cells. The cellular consequences of N-BP exposure include anti-tumor activity, cytoskeletal disruption, renal toxicity, endothelial dysfunction, and alterations in lipid and energy metabolism. Many of these effects may be related to mitochondrial dysfunction, including impaired respiratory function, impaired calcium homeostasis, deficiencies in CoQ and heme *a* in mitochondria and activation of mitochondria-dependent apoptosis. Despite the growing awareness of these off-target effects, the impact of N-BPs at the mitochondrial level remains a largely unexplored area of research. In particular, the role of mitochondrial CoQ deficiency as a contributor to N-BP toxicity is understudied, and the potential for CoQ supplementation to ameliorate these effects has not been investigated.

Future perspectives should include an in-depth exploration of N-BP-induced mitochondrial dysfunction, with a focus on respiratory chain function and mitochondrial quality control mechanisms. Particular attention should be paid to the evaluation of CoQ supplementation as a potential protective strategy to ameliorate mitochondrial impairment and disruption of cellular energy metabolism. Further studies on the complex interplay between the mevalonate pathway, mitochondrial function, and overall cellular homeostasis will be important to optimize the clinical use of N-BPs while reducing unintended effects on non-target tissues.

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