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Polimeraza poli(ADP-rybozy) w starzeniu, neurotoksyczności peptydów amyloidu beta oraz w patologii niedokrwiennej mózgu

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Warszawa, listopad 2005

SPIS TREŚCI

I. WSTĘP

1. Informacje ogólne: budowa i znaczenie fizjologiczne PARP-1

Polimeraza poli(ADP-rybozy), PARP-1, (EC 2.4.2.30), jest jednym z najlepiej scharakteryzowanych enzymów, biorących udział w naprawie uszkodzeń DNA i regulacji procesów transkrypcji. PARP-1 jest białkiem monomerycznym, zlokalizowanym głównie w jądrze komórki (ponad 90%), o masie cząsteczkowej 113 kDa u ssaków i w granicach 110-130 kDa u innych gatunków. PARP-1 jest najlepiej poznanym członkiem rodziny PARP, w skład której wchodzą również: sPARP-1, PARP-2, PARP-3, PARP-4 (VPARP), PARP-5a (tankyraza-1), PARP-5b (tankyraza-2), PARP-6, PARP-7, PARP-8 (Chiarugi 2002, Hassa i Hottiger 2002). W komórkach ośrodkowego układu nerwowego PARP-1 odpowiada za ponad 90% zachodzących procesów poli(ADP-rybozylacji) (Pieper i wsp. 2000). Enzym ten ma trzy istotne strukturalne domeny: N-końcową domenę, która wiąże DNA (42-46 kDa, DNA binding domain, DBD) i zawiera 2 palce cynkowe, centralną domenę automodyfikującą 16-22 kDa, zawierającą motyw BRCT odpowiedzialny za interakcje na poziomie białko-białko, i C-końcową domenę katalityczną o masie cząsteczkowej 54 kDa. Domena katalityczna jest odpowiedzialna za przekształcenie NAD^+ do amidu kwasu nikotynowego i cząsteczki ADP-rybozy, która jest dołączana do PARP-1 (proces autorybozylacji) oraz do innych białek jądrowych. Jest to najbardziej konserwatywna domena ze wszystkich trzech.

Poza omówionym powyżej PARP-1, najwcześniej zidentyfikowanym, obecnym również w jądrze komórki enzymem z tej rodziny, jest PARP-2. Białko to (62 kDa) wykazuje prawie 70% stopień homologii z PARP-1. Funkcja PARP-2 nie jest dokładnie poznana. Uważa się, że modyfikuje on chromatynę, wchodzi ponadto w interakcję z PARP-1 i współdziałając między innymi z DNA polimerazą β i DNA ligazą III, bierze udział w naprawie jednoniciowych pęknięć DNA. PARP-2 wykazuje ograniczoną aktywność w syntetyzowaniu polimerów poli(ADP-rybozy). Badania nad znaczeniem PARP-2, z wykorzystaniem myszy pozbawionych genu dla PARP-2, wykazały, że funkcje PARP-1 i PARP-2 są w wielu przypadkach komplementarne, ale nie pokrywają się w pełni. PARP-3 charakteryzuje najmniejsza spośród wszystkich PARP-ów domena N-końca, w której znajduje się lokalizacja centrosomalna (Augustin i wsp. 2003). Stopień homologii pomiędzy PARP-3 a

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PARP-1 wynosi około 60%. Wiadomo też, że PARP-3 wchodzi w interakcje z PARP-1, którego obecność stwierdzono również w centrosomach (Kanai i wsp. 2003). PARP-4 (VPARP) jest największym białkiem z rodziny polimeraz poli(ADP-rybozy), o ciężarze cząsteczkowym 192 kDa. Funkcja tego białka jest w zasadzie nieznana. Przypisuje się mu udział w transporcie komórkowym. Stwierdzono jego obecność w porach błony jądrowej i we wrzecionie mitotycznym podczas mitozy. PARP-5a, czyli tankyraza 1, został zidentyfikowany jako białko towarzyszące telomerom (Smith i wsp. 1998). Stwierdzono, że nadekspresja tankyrazy może umożliwiać wydłużanie się telomerów (Smith i de Lange 2000). PARP-5b, czyli tankyraza-2, został zidentyfikowany przez Kaminkera i wsp. (2001). Białko to wykazuje ponad 85% stopień homologii z tankyrazą 1. Obie tankyrazy współdziałają ze sobą oraz z tą samą grupą białek i prawdopodobnie ich funkcje się pokrywają w zakresie homeostazy telomerów i transportu pęcherzykowego. Oba enzymy występują w licznych strukturach podkomórkowych: w telomerach, porach jądrowych, pęcherzykach Golgiego, pericentriolach podczas mitozy. Nadekspresja tankyrazy 2 może powodować niezależną od kaspaz śmierć komórki (Kaminker i wsp. 2001). PARP-7 prawdopodobnie odgrywa istotną rolę w funkcji komórek T oraz w procesach nowotworzenia. Posiada w swojej strukturze tylko jeden palec cynkowy i domenę, która występuje w białkach podlegających ADP-rybozylacji.

Pozostałe polimerazy poli(ADP-rybozy), sekwencjonowane z ludzkiego genomu, nie są do chwili obecnej dokładnie poznane. Określona została ich struktura, natomiast niewiele wiadomo o ich funkcji. Interesującą właściwością enzymów białek rodziny PARP jest ich zdolność do współdziałania ze sobą, tak np. PARP-1 z PARP-2 (Schreiber i wsp. 2002) i z PARP-3 (Augustin i wsp. 2003) czy tankyraza 1 z tankyrazą 2 (Cook i wsp. 2002, Sbodio i wsp. 2002). Tego typu interakcje mogą zwiększać ich znaczenie biologiczne.

Warto tu jeszcze raz podkreślić, iż wyłącznie PARP-1 i PARP-2 posiadają domenę, wiążącą się z DNA. PARP-1 posiada zdolność rozpoznawania jedno i dwuniciowych uszkodzeń DNA i bierze udział w naprawie DNA przez kompleks BER (base excission repair - usuwanie uszkodzonych zasad) oraz przez kompleks NER (nucleotide excission repair - usuwanie uszkodzonych nukleotydów).

PARP powoduje ADP-rybozylację licznych białek jądrowych, przenosząc do 200 molekuł ADP-rybozy. W ciągu kilkudziesięciu sekund od utworzenia polimerów poli(ADP-ryboza) jest hydrolizowana przez glikohydrolazę poli(ADP-rybozy) (PARG EC 3.2.1.143) do ADP-rybozy. Poli(ADP-rybozylacji) ulegają histony,
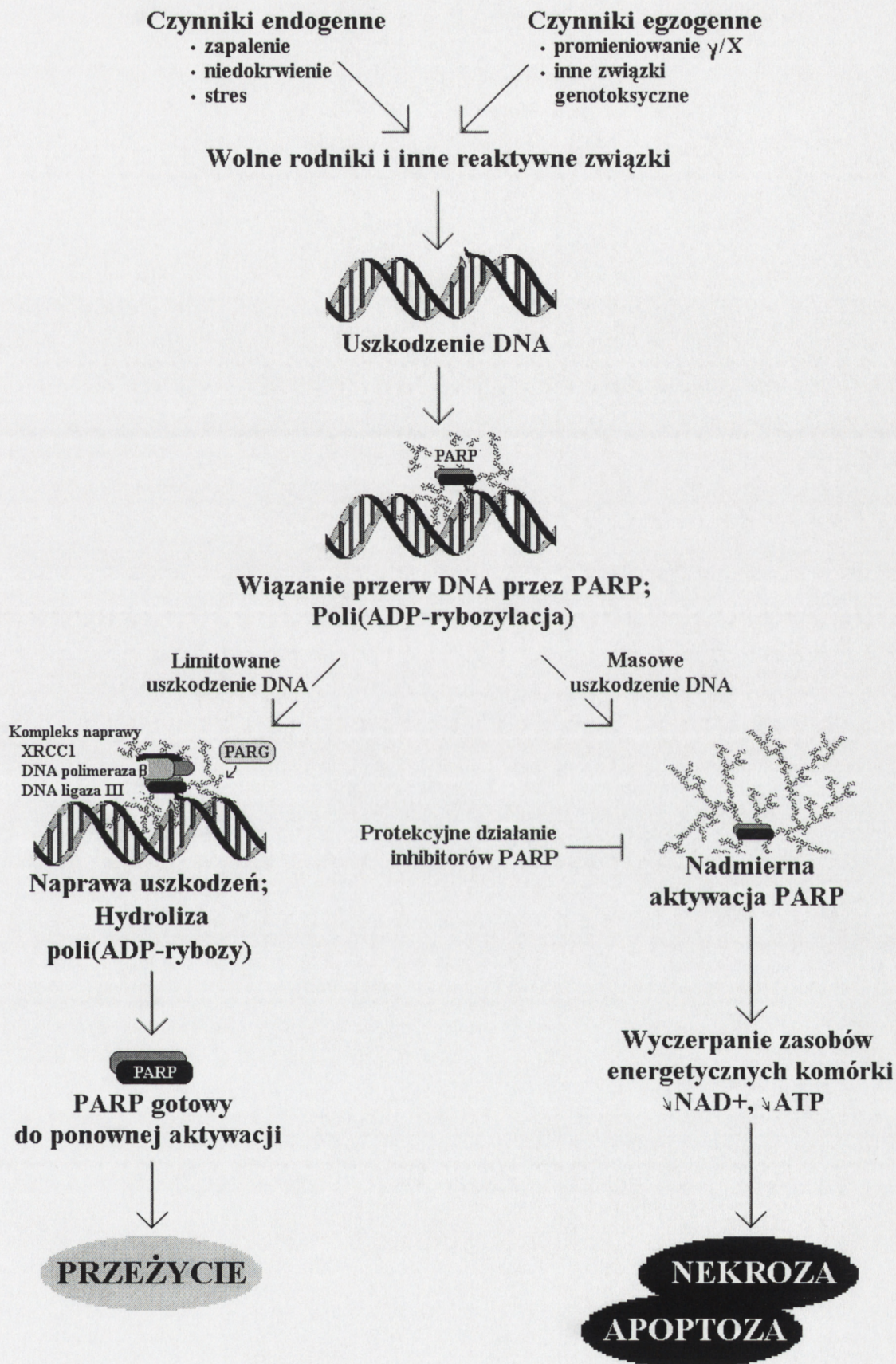
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polimerazy DNA 1 i 2, ligazy DNA, topoizomerazy, Ca^{2+}/Mg^{2+} zależna endonukleaza i sam PARP-1, który ulega autorybozylacji (Ueda i wsp.1982, Ueda i Hayaishi, 1985, Althaus, Richter, 1987, Lautier i wsp. 1993, de Murcia i wsp. 1994, D'Amours i wsp. 1999, Koh i wsp. 2005b).

Biologiczną rolę PARP można zawrzeć w 6 punktach:

1. udział w reperacji DNA i utrzymaniu stabilności genomu
2. udział w procesach obumierania komórki na drodze nekrozy lub apoptozy
3. regulacja procesów transkrypcji
4. regulacja procesów replikacji i różnicowania
5. regulacja aktywności telomeraz
6. funkcje regulacyjne w organizacji cytoskeletonu komórki

PARP-1 katalizuje powstawanie, wydłużanie i rozgałęzienie polimeru poli(ADP-rybozy) (PAR), jak również rybozyluje DNA-kinazę, enzym zaangażowany w naprawę podwójnych pęknięć nici DNA, współodpowiedzialny za stabilność genomu. Proces rybozylacji powoduje wzrost aktywności DNA-kinazy. Ostatnio sugeruje się, że DNA-kinaza może fosforylować PARP-1 i hamować jego aktywność. Stwierdzono ponadto, że PARP-1 za pośrednictwem ADP-rybozylacji utrzymuje w stanie nieaktywnym wapniowo-magnezowo zależną endonukleazę. W reakcji ADP-rybozylacji amid kwasu nikotynowego uwalniany jest jako półprodukt i zwrotnie może hamować enzym. Proces ten zachodzi podczas reperacji DNA.

Jednak trzeba wyraźnie podkreślić, że "pomoc" PARP w reperacji nici DNA jest możliwa tylko w przypadku ograniczonego uszkodzenia DNA. Przy masywnym uszkodzeniu DNA następuje nadmierna aktywacja PARP, prowadząca do wyczerpania βNAD^+, będącego donorem grup ADP-rybozy. W procesie resyntezy βNAD^+ następuje wyczerpanie ATP prowadzące do śmierci komórki (Ryc. 1).

Czynniki endogenne
- zapalenie
- niedokrwienie
- stres

Czynniki egzogenne
- promieniowanie γ/X
- inne związki genotoksyczne

Wolne rodniki i inne reaktywne związki

Uszkodzenie DNA

PARP

**Wiązanie przerw DNA przez PARP;
Poli(ADP-rybozylacja)**

Limitowane uszkodzenie DNA

Masowe uszkodzenie DNA

Kompleks naprawy
XRCC1
DNA polimeraza β
DNA ligaza III

PARG

**Naprawa uszkodzeń;
Hydroliza
poli(ADP-rybozy)**

Protekcyjne działanie inhibitorów PARP

**Nadmierna
aktywacja PARP**

PARP

**PARP gotowy
do ponownej aktywacji**

**Wyczerpanie zasobów
energetycznych komórki
↓NAD+, ↓ATP**

PRZEŻYCIE

NEKROZA

APOPTOZA

Ryc. 1. Udział PARP-1 w przeżyciu i śmierci komórki w modyfikacji wg Jeggo (1998)

Poli(ADP-ryboza) (PAR) jest głównie zlokalizowana w jądrze. Katabolizm PAR jest aktywowany przez poli(ADP-rybozo) glikohydrolazę (PARG).

Obecnie zidentyfikowano trzy izoenzymy PARG. Czas półtrwania PAR różni się w zależności od długości łańcucha polimeru oraz natury białka akceptorowego. Wyniki badań wskazują, że wynosi on poniżej 1 min. Badania ostatnich lat wykazały, że PARG bierze udział w regulacji cyklu komórkowego, w formowaniu wrzeciona mitotycznego oraz w rozwoju organizmu (Ohashi i wsp. 2003, Hanai i wsp. 2004, Chiarugi 2005, Koh i wsp. 2005a). Krótki czas trwania PAR sugeruje wysokie obroty metaboliczne polimeru w nieuszkodzonych komórkach. Te procesy z kolei mogą wyjaśnić, dlaczego aktywowanie biosyntezy PAR wywołuje znaczący spadek stężenia βNAD^+. Obserwacje te wpłynęły na powstanie teorii samobójczej śmierci komórki Bergera (1985).

2. PARP-1 a fosfolipidy inozytolowe i lipidowe przekaźniki informacji

Badania ostatnich lat (Homburg i wsp. 2000, Cohen-Armon i wsp. 2005) wykazały, że w warunkach fizjologicznej stymulacji, np. podczas depolaryzacji, degradacja fosfolipidów inozytolowych przez fosfolipazę C (PLC) i uwalnianie inozytolo(1,3,5)trisfosforanu (IP$_3$) bierze udział w przekazywaniu sygnału do PARP-1 powodując jego aktywację. Stwierdzono, że zależna od IP$_3$ wewnątrzkomórkowa mobilizacja jonów wapnia uczestniczy w przekazywaniu informacji do PARP-1, przy zachowaniu integralności DNA. Ponadto wykazano, że inhibitor receptora dla IP$_3$, TMB-8 eliminuje aktywację PARP-1, wywołaną pobudzeniem układu cholinergicznego (Strosznajder i wsp. 2005a). Wyniki powyższe wskazują na istotną zależność pomiędzy metabolizmem fosfolipidów inozytolowych, a aktywnością PARP-1. Istnieją również dane literaturowe (Cohen-Armon i wsp. 2005), wskazujące na udział procesów fosforylacji-defosforylacji w regulacji aktywności PARP-1. Uwalniany przez fosfolipazę C diacyloglicerol (DAG), jako istotny aktywator kinazy białkowej C (PKC), może brać udział w regulacji aktywności PARP-1. Wstępne badania wykazały, że PKC nie bierze udziału w regulacji konstytutywnej aktywności PARP-1 (Strosznajder i wsp. 2005a). Natomiast wyniki badań Cohen-Armon i wsp. (2005) wskazują na istotne znaczenie w aktywacji PARP-1 kinazy ERK2.

Ponadto wykazano w badaniach *in vitro* na wyizolowanym enzymie, że kwas arachidonowy (KA), istotny lipidowy przekaźnik I i II rzędu, hamuje aktywność PARP (Banasik i Ueda 1994). Należy jednak zauważyć, że KA, uwalniany w okresie

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przywrócenia krążenia po niedokrwieniu mózgu, może brać udział w kaskadzie wolnorodnikowej i tą drogą aktywować PARP-1.

3. PARP-1 a czynniki transkrypcyjne

PARP-1 odgrywa kluczową rolę w regulacji aktywności licznych czynników transkrypcyjnych. Enzym ten może wpływać na aktywność transkrypcyjną poprzez interakcje na poziomie białko-białko lub też poprzez swoją aktywność katalityczną, dzięki której może nastąpić proces poli(ADP-rybozylacji) czynników transkrypcyjnych.

Czynniki transkrypcyjne, takie jak AP-2 (Kannan i wsp. 1999), B-MYB (Cervellera i Sala 2000), Oct-1 (Nie i wsp. 1998), YY-1 (Oei i wsp. 1997) i TEF-1 (Butler i Ordahl 1999), zostały zidentyfikowane, jako wchodzące w bezpośrednią interakcję z PARP-1. Z badań Griesenbecka i wsp. (1999) wiadomo, że interakcja PARP z czynnikiem transkrypcyjnym YY-1 może stymulować aktywność PARP-1 prawie dziesięciokrotnie. Z drugiej strony, czynniki takie jak p53 (Wesierska i Schmid 2001) i c-fos (Amstad i wsp. 1992) są poli(ADP-rybozylowane).

Transkrypcyjna aktywacja NF-kappaB spowodowana stresem/zapaleniem ulega obniżeniu w komórkach pozbawionych PARP-1 (Oliver i wsp. 1999, Hassa i Hottiger 1999). PARP-1 bierze udział w aktywacji NF-kappaB poprzez wiązanie bezpośrednie do tego czynnika transkrypcyjnego (Hassa i wsp. 2001, Urlich i wsp. 2001) lub też poprzez jego poli(ADP-rybozylację) (Kameoka i wsp. 2000, Chang i Alvarez-Gonzalez 2001). Również dane uzyskane przez Oliver i wsp. (1999), Hassa i wsp. (2001) oraz Le Page i wsp. (1998) wskazują, że aktywacja NF-kappaB zależy od PARP-1.

Badania, które pokazują bezpośrednie łączenie PARP-1 do czynników transkrypcyjnych, takich jak Oct-1 (Nie i wsp. 1998) i YY-1 (Oei i wsp. 1997), sugerują nową rolę PARP jako koaktywatora czy też represora czynników transkrypcyjnych, biorących udział w zapaleniu czy stresie. Poprzednio uważano, że aktywacja PARP-1 wywołana zapaleniem/stresem jest powiązana tylko z NF-kappaB (La Page i wsp. 1998, Oliver i wsp. 1999, Hassa i wsp. 2001). Obecne badania wskazują, że aktywacja procesów, związanych ze stresem wywołanym podaniem np. lipopolisacharydu (LPS) czy TNF-α, łączy współdziałanie PARP-1 z takimi czynnikami transkrypcyjnymi, jak AP-1, SP-1, Oct-1, YY-1 i Stat-1. Wiadomo także, że brak PARP hamuje ekspresję TNF-α, interferonu-γ oraz iNOS, jak również

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wewnątrzkomórkowej adhezyjnej molekuły-1 (Zingarelli i wsp. 1998, Oliver i wsp. 1999, Decker i Muller 2002, Virag i Szabo 2002).

Do chwili obecnej molekularny mechanizm regulacji p53 i NF-kappaB przez PARP-1 nie jest całkowicie zrozumiały, a jądrowa interakcja między tymi białkami jest przedmiotem dalszych intensywnych badań (Hassa i Hottiger 1999, Chiarugi 2005). Białko p53 jest czynnikiem transkrypcyjnym, który reguluje cykl komórkowy, wpływa na proliferację komórki i indukuje apoptozę przez aktywację programu genetycznego. Wiele danych wskazuje, że PARP-1 jest bardzo ważnym modulatorem programowanej śmierci komórki wywołanej przez p53. PARP-1 i białko p53 zwiększają swoją aktywność w odpowiedzi na stres genotoksyczny (Juarez-Salinas i wsp. 1979, Berger 1985, Oren 1992, Lane 1992). Oba białka biorą udział w naprawie DNA (Kastan i wsp. 1991, Lane 1992) i oba białka mogą być odpowiedzialne za zaburzenia cyklu komórkowego w wyniku uszkodzenia DNA (Borrstein i Pardee 1984, Oren 1992, Kuerbitz i wsp. 1992). Nadmierna aktywacja PARP i nadekspresja p53 prowadzą do obumierania komórek. Aktywność PARP i synteza poli(ADP-rybozy) wzrasta wielokrotnie w sekundach lub minutach po uszkodzeniu DNA, podczas gdy wzrost poziomu p53 jest zdecydowanie wolniejszy. Komórki z obniżoną poli(ADP-rybozylacją) mają niższy poziom aktywności p53 i są mniej wrażliwe na zależną od p53 apoptozę (Whitacre i wsp. 1995). Poli(ADP-rybozylacja) jest konieczna dla ekspresji p53 (Agarwal i wsp. 1997, Wang i wsp. 1998). Fizyczna interakcja pomiędzy PARP-1 a p53 była prezentowana i stwierdzono, że p53 jest akceptorem łańcuchów ADP-rybozy, które przyłączają się do specyficznych domen p53 i zmieniają jego zdolności do wiązania się z DNA (Malanga i wsp. 1998). Aktywacja NF-kappaB i p53 była obserwowana w neuronach, poddanych działaniu ekscytotoksycznych stężeń glutaminianu w ostrych i chronicznych procesach neurodegeneracji (Grilli i Memo 1999, Uberti i wsp. 2000) oraz w niedokrwieniu mózgu (Li i wsp. 1994). Aktywacja NF-kappaB jest istotna zarówno dla ekspresji p53, jak również jego aktywności (Kirch i wsp. 1999). Zależność pomiędzy p53 a NF-kappaB jest bardzo istotna dla regulacji życia i śmierci komórki.

Dotychczasowe badania nad udziałem białka p53 w procesach przeżycia i obumierania neuronów, w patologii niedokrwiennej mózgu są kontrowersyjne i wymagają dalszych wyjaśnień. Badania Maedy i wsp. (2001) oraz Tomasevica i wsp. (1999a i 1999b) wskazują, że p53 raczej zapobiega aniżeli nasila uszkodzenie mózgu wywołane niedokrwieniem. Natomiast badania Culmsee (2002, 2003, 2005)

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wykazały, że białko p53 jest odpowiedzialne za śmierć komórek w wyniku niedokrwienia mózgu i że może być znakomitym punktem uchwytu dla neuroprotekcyjnych związków.

Mniej kontrowersji dotyczy udziału NF-kappaB w mechanizmie tolerancji na niedokrwienie mózgu. Badania Morgana i wsp. (1999) wykazały, że aktywacja NF-kappaB jest konieczna dla cytoprotekcyjnego efektu hartowania ischemicznego. Dane uzyskane przez Blondeau i wsp. (2001) wskazują, że w mechanizmie hartowania ischemicznego dochodzi do hamowania aktywacji NF-kappaB poprzez stymulację transkrypcji IkBα i to prawdopodobnie przez sam NF-kappaB. Zingarelli i wsp. (2003) po raz pierwszy pokazali, iż u zwierząt pozbawionych PARP uzyskana kardioprotekcja jest powiązana z obniżeniem aktywności NF-kappaB. Badacze stwierdzili, że obniżenie aktywności NF-kappaB u myszy pozbawionych PARP było powiązane z inhibicją degradacji IkBα i obniżeniem aktywności IKK (Zingarelli i wsp. 2003).

Zaobserwowano także, że poziom antyapoptotycznego białka Bcl-2 obniża się w okresie przywrócenia krążenia u szczepów dzikich (PARP$^{+/+}$), natomiast wyraźnie wzrasta poziom Bcl-2 u myszy pozbawionych genu dla PARP (PARP$^{-/-}$). Badanie ekspresji proapoptotycznego białka Bax wykazało, iż jego poziom pozostaje niezmieniony po niedokrwieniu, jak i w okresie przywrócenia krążenia w obu grupach badanych zwierząt (Zingarelli i wsp. 2003). Stwierdzono również, że proapoptotyczna aktywność kaspaz wzrastała wyraźnie pod koniec niedokrwienia, powracając do podstawowej aktywności po pierwszej godzinie przywrócenia krążenia u zwierząt posiadających gen dla PARP. Natomiast u myszy pozbawionych tego genu (PARP$^{-/-}$) zaobserwowano obniżenie aktywności, zarówno kaspazy 1 jak i 3, po zastosowaniu 30 minutowego niedokrwienia mięśnia sercowego (Zingarelli i wsp. 2003). Badania Xuan i wsp. (1999) dokumentują udział NF-kappaB w późnej fazie tolerancji na niedokrwienie. Potencjalnym mechanizmem w zależnym od PARP-1 obumieraniu neuronów może być zmiana jego interakcji z czynnikami transkrypcyjnymi NF-kappaB i p53 (Oliver i wsp. 1999, Chiarugi 2002, Chiarugi i Moskowitz 2003). W wyniku stresu oksydacyjnego, wywołanego przywróceniem krążenia po niedokrwieniu mózgu i procesami zapalnymi, aktywacja PARP-1 może prowadzić do aktywacji NF-kappaB, co z kolei powoduje indukcję genu dla iNOS i cytokin oraz śmierć komórek (Hassa i Hottiger 1999).

Warto tu jeszcze raz podkreślić, że PARP-1 może działać poprzez bezpośrednie łączenie się z czynnikami transkrypcyjnymi jako koaktywator czy

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represor, w sposób niezależny od aktywności swojej domeny katalitycznej. Ostatnie dane wskazują, że wiązanie PARP-1 do DNA nie jest wystarczające samo w sobie dla spowodowania ekspresji genów, natomiast odpowiedni poziom poli(ADP-rybozy) wydaje się konieczny dla aktywności transkrypcyjnej, o czym wspomniano już uprzednio.

4. Polimeraza poli(ADP-rybozy) w procesie starzenia

W połowie lat osiemdziesiątych grupa Pero i wsp. (1985) oceniała aktywność podstawową (konstytutywną) enzymu, powiązaną ze wzrostem i proliferacją komórki, jak również aktywność PARP po stymulacji promieniowaniem γ, powiązaną z reperacją uszkodzonej nici DNA. Do badań użyto leukocyty jednojądrzaste, pochodzące od 12 gatunków ssaków. Aktywność konstytutywna enzymu w badaniach Pero i wsp. (1985) była bardzo zbliżona u szczura, myszy, owcy, goryla, szympansa i człowieka. Natomiast u słonia była kilkukrotnie wyższa. Autorzy stwierdzili wysoki współczynnik korelacji pomiędzy stymulowaną aktywnością PARP a długością życia u badanych ssaków (r=0.97). Potwierdzenie wysokiej korelacji pomiędzy aktywnością PARP a długością życia uzyskali również uczeni niemieccy Grübe i Bürkle (1992), w badaniach komórek jednojądrzastych krwi (limfocytów, monocytów/makrofagów) u 13 gatunków ssaków (r=0.84). Natomiast Jackowsky i Kun (1981) stwierdzili niższy poziom PARP w 90-dniowych komórkach mięśnia serca, w porównaniu z komórkami 5-dniowymi. Nie zaobserwowano wyraźnej korelacji pomiędzy ilością białka (PARP) a długością życia. Nie stwierdzono wyraźnej różnicy pomiędzy ilością białka u szczura i człowieka, przy istotnej różnicy w jego specyficznej aktywności.

Muiras i wsp. (1998) badali poziom aktywności PARP w populacji osób z terenu Francji, ze średnią wieku stu lat, na materiale unieśmiertelnionych linii komórek limfoblastów. Stwierdzono wyraźnie statystycznie wyższą aktywność enzymatyczną u stulatków, w porównaniu z grupą kontrolną (wiek 20-70 lat), w przeliczeniu na ilość komórek. Badania wykazały również brak statystycznie znamiennych różnic w ilości białka pomiędzy stulatkami a grupą kontrolną (Muiras i wsp. 1998). Porównując specyficzną aktywność PARP w przeliczeniu na białko enzymu w obu grupach, Muiras i wsp. (1998) stwierdzili wysoką, znamiennie statystyczną różnicę pomiędzy stulatkami a grupą kontrolną. Wartość mediany uzyskanej z linii komórkowej stulatków była 1,6 razy wyższa od wartości kontrolnych. Wysunięto hipotezę, że zmiana specyficznej aktywności PARP

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pomiędzy stulatkami a grupą kontrolną, jest spowodowana różnicami jakościowymi białka enzymu, prawdopodobnie uwarunkowanymi genetycznie na poziomie kodowanej przez gen PARP sekwencji aminokwasowej białka enzymu. Ta sugestia może podbudowywać hipotezę o udziale komponenty genetycznej, warunkującej długowieczność (Abbot i wsp. 1974; Schachter i wsp. 1993). Prowadzone badania nie są w stanie wykluczyć posttranslacyjnej modyfikacji enzymu lub jego interakcji z niezbadanymi obecnie składnikami jądra komórki, warunkującymi zwiększenie jego zdolności do pełnienia funkcji strażnika stabilności genomu, jak sugeruje Jeggo (1998).

Rozważając problemy starzenia się w aspekcie zmian w aktywności PARP, warto zacytować pracę Bizec i wsp. (1989), w której badano wpływ wieku na aktywność PARP, jak i na aktywność poli(ADP-rybozo) glikohydrolazy (PARG), enzymu biorącego udział w degradacji poli(ADP-rybozy). Badania przeprowadzano na komórkach nabłonka soczewki wołu, w 3 grupach doświadczalnych (23 miesiące -młode, 54 miesiące - dorosłe, 94 miesiące - stare). Stwierdzono malejącą aktywność PARG wraz z upływem wieku, w przeciwieństwie do aktywności PARP, która wzrastała znacząco. Ponadto Bizec i wsp. (1989) zaobserwowali zwiększenie liczby łańcuchów poli(ADP-rybozy) (PAR) w trakcie starzenia. Autorzy uważają, że wzrost aktywności PARP w starzeniu jest prawdopodobnie spowodowany zwiększoną liczbą uszkodzeń nici DNA. W późniejszej pracy Messripour i wsp. (1994) wykazali około trzykrotny wzrost aktywności PARP, któremu towarzyszył wzrost ilości białka enzymu w populacji neuronów i astrocytów z całego mózgu 30-miesięcznych szczurów, w porównaniu z 3-miesięczną grupą kontrolną.

Kolejne badania wykazały wzrost poziomu PAR w chorobach neurodegeneracyjnych, w chorobie Alzheimera oraz Parkinsona. Należałoby jednak rozróżnić wzrost aktywności PARP i poziomu PAR w procesie starzenia i w chorobie Alzheimera. W procesie starzenia PARP miałby pełnić funkcję strażnika stabilności genomu i wzrost jego aktywności miałby świadczyć o stanie gotowości enzymu do naprawy nici DNA. W chorobie Alzheimera wzrost aktywności PARP jest wyrazem znacznego uszkodzenia nici DNA. Love i wsp. (1999) badali zmiany poziomu PARP w mózgach ludzi z chorobą Alzheimera. Wykazali oni w badaniach immunohistochemicznych, przeprowadzonych na materiale autopsyjnym, że zarówno sam PARP, jak i poziom PAR znacznie wzrastał w korze czołowej i skroniowej osób z chorobą Alzheimera, w porównaniu do odpowiednich kontroli wiekowych. Związane to jest najprawdopodobniej ze znacznym wzrostem działania

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wolnych rodników i uszkodzeniem nici DNA. Nadmierna aktywacja enzymu miałaby w tym przypadku prowadzić do niekontrolowanej ADP-rybozylacji białek oraz wyczerpania βNAD^+ i ATP, a w konsekwencji do obumierania neuronów.

5. Rola PARP-1 w niedokrwieniu

Znaczenie polimerazy poli(ADP-rybozy) w patologii niedokrwiennej mózgu było i jest przedmiotem intensywnych badań. Można je podzielić na badania nad niedokrwieniem lokalnym/ogniskowym, gdzie uzyskane wyniki z inhibitorami PARP nie budzą dziś większych kontrowersji, oraz badania nad niedokrwieniem globalnym, gdzie sprzeczne wyniki wymagają prowadzenia dalszych badań.

Takahashi i wsp. (1997) w badaniach przeprowadzonych in vivo stwierdzili, że podanie inhibitorów PARP prowadzi do znamiennego zmniejszenia powierzchni zawału w modelu miejscowego niedokrwienia mózgu u szczura. Badania wykonywane na myszach pozbawionych PARP-1 wskazywały, że brak PARP daje protekcję w zwierzęcym modelu ogniskowego niedokrwienia mózgu (Eliasson i wsp. 1997, Endres i wsp. 1997). W przypadku niedokrwienia mózgu badania z użyciem myszy, posiadających gen dla PARP, wykazały, że neuroprotekcyjny efekt stosowania 3-aminobenzamidu (3-AB), inhibitora PARP, był podobny do wyników uzyskanych u zwierząt pozbawionych genu dla PARP (Endres i wsp. 1997). Wyniki badań Lo i wsp. (1998) pokazują, że inhibicja farmakologiczna PARP znacząco zmniejsza obszar zawału, spowodowany zamknięciem tętnicy środkowej mózgu. Ci sami badacze (Lo i wsp. 1998) stosując mikrodializę stwierdzili, że perfuzja z NMDA powodowała wyraźny wzrost glutaminianu, tauryny oraz fosfoetanolaminy. Natomiast podanie 3-AB znamiennie obniżało wzrost glutaminianu i fosfoetanolaminy. Thiemermann i wsp. (1997) zaobserwowali, że podanie dotętniczo 3-AB przed wywołaniem niedokrwienia powodowało zmniejszenie obszaru zawału mięśnia sercowego o ponad 40% oraz częściowo likwidowało dysfunkcje tego mięśnia bez zmian parametrów hemodynamicznych zwierzęcia. W badaniach stosowano również amid kwasu nikotynowego, który działał kardioprotekcyjnie. Jako kontrolę zastosowano kwas nikotynowy, który nie dawał protekcji. Autorzy wskazują, że tak jak chroniczna inhibicja PARP może prowadzić do licznych efektów ubocznych, tak przejściowa inhibicja tego enzymu może stanowić terapeutyczną drogę w przypadku epizodów niedokrwiennych (Thiemermann i wsp. 1997, Thiemermann i wsp. 2005).

Fiorillo i wsp. (2003) badali wpływ inhibitora PARP, 3-AB w doświadczeniach z przeszczepem serca. Stwierdzono, że podanie 3-AB, zarówno dawcy jak i biorcy, znacząco obniża poziom stresu oksydacyjnego, podtrzymując poziom NAD^+ i ATP. Również poziom nieuszkodzonego DNA w sercach poddanych transplantacji, po podaniu 3-AB był znacząco wyższy w porównaniu z kontrolą. Podanie 3-AB obniża poziom znaczników, mówiących o uszkodzeniu mięśnia sercowego. Zmniejszeniu ulega poziom dialdehydu malonylowego (MDA) jak i 4-hydroksynonenalu (4-HNE) w mięśniu sercowym, podlegającym transplantacji, w stosunku do tkanki kontrolnej (Fiorillo i wsp. 2003).

Podczas niedokrwienia mózgu dochodzi do nadmiernego wzrostu stężenia glutaminianu i aktywacji receptorów glutaminianergicznych typu NMDA (N-methyl-D-aspartate). Stwierdzono, że PARP odgrywa kluczową rolę w ekscytotoksyczności receptora NMDA, natomiast nie bierze udziału w ekscytotoksyczności innych receptorów glutaminianergicznych, np., AMPA (Mandir i wsp. 2000). Badania prowadzono, stosując stereotaktyczne podanie NMDA do prążkowia myszy, pozbawionych genu dla PARP-1, i porównywano wyniki z myszami kontrolnymi po 48 godzinach, w doświadczeniach ostrych oraz po upływie 3 tygodni w doświadczeniach chronicznych. Myszy pozbawione PARP-1 okazały się wysoce oporne na ekscytotoksyczność, wywołaną NMDA, natomiast pozostawały wrażliwe na ekscytotoksyczność AMPA, podobnie jak szczepy dzikie. Podanie genu dla białka PARP-1 myszom, pozbawionym tego genu, w procesie transfekcji wirusowej, przywracało wrażliwość na NMDA i potwierdzało istotną rolę PARP-1 w neurotoksyczności NMDA. Stwierdzono ponadto, że NMDA nie powoduje aktywacji PARP u myszy, pozbawionych genu dla nNOS. Wyniki tych badań dodatkowo potwierdziły specyficzną, selektywną rolę PARP-1 w neurotoksyczności, wywołanej pobudzeniem receptora NMDA, i wskazują na nowe możliwości neuroprotekcyjnego działania inhibitorów PARP. Aktywacja PARP poprzez receptor NMDA jest podobna do obserwowanej aktywacji tego enzymu po zamknięciu światła tętnicy środkowej mózgu (Elliasson i wsp. 1997, Endres i wsp. 1997, Lo i wsp. 1998, Tokime i wsp. 1998).

Stwierdzono, że pozbawione genu PARP-1 myszy są oporne na ogniskowe niedokrwienie mózgu. Należy zaznaczyć, że neuroprotekcja obserwowana u myszy, pozbawionych genu dla PARP, jest dużo wyższa, niż neuroprotekcja uzyskiwana u myszy, pozbawionych genu dla nNOS. Wskazuje to, że PARP-1 jest aktywowany również przez inne cytotoksyczne mechanizmy, nie związane z procesami

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wolnorodnikowymi zależnymi od NO/ONOO⁻ (Skaper 2003). W modelu globalnego niedokrwienia Traystman i wsp. (2002) stwierdzili istotną rolę PARP w obumieraniu neuronów. Swanson (2002) zwrócił uwagę, że wzrost aktywności PARP zaburza wychwyt glutaminianu przez astrocyty i tym samym zwiększa uszkodzenie neuronów w niedokrwieniu mózgu. Podczas niedokrwienia neurony uwalniają Zn^{2+} i NO, które to związki zapoczątkowują uszkodzenie DNA i aktywację PARP. W hodowli astrocytów stwierdzono, że hamowanie PARP przez benzamid zapobiega zaburzeniom wychwytu glutaminianu w wyniku działania Zn^{2+} i NO. Uważa się więc, że PARP pełni istotną rolę w zaburzeniu funkcji nie tylko neuronów, ale również astrocytów po niedokrwieniu. Warto tu nadmienić, że PARP jest znacząco aktywowany w fagocytujących komórkach mikrogleju, pomimo jego niewielkiego poziomu w mikrogleju spoczynkowym. Tak więc obniżenie jego aktywności może mieć istotne znaczenie w protekcji komórek neuronalnych (Ullrich i wsp. 2001).

Należy przypomnieć, że PARP-1 jest ponad 70-krotnie bardziej aktywny w neuronach w porównaniu z jego aktywnością w komórkach glejowych (Pieper i wsp. 2000). Natomiast badania glikohydrolazy poli(ADP-rybozy) (PARG) w neuronach i astrocytach wykazały zdecydowanie wyższy poziom aktywności PARG w cytoplazmie neuronów w porównaniu z ich frakcją jądrową. Aktywność PARG w astrocytach była podobna w obu badanych frakcjach (Sevigny i wsp. 2003). Dane przedstawione przez Lu i wsp. (2003) pokazały, że inhibitor PARG, o symbolu GPI 16552, ma właściwości neuroprotekcyjne w modelu przejściowego niedokrwienia u szczura. Cozzi i wsp. (2005) w badaniach z użyciem myszy ($PARG_{110}^{-/-}$) stwierdził obniżoną ekspresję dla białka szoku termicznego HSP-70 i wzrost ekspresji dla cyklooksygenazy-2. Wyniki te wskazują, że zaburzony katabolizm PAR zwiększa uszkodzenia w mózgach, poddanych niedokrwieniu. W uwzględnianiu roli PARG w patologii niedokrwiennej należy mieć na uwadze jego znaczenie w regulacji ekspresji genów (Rapizzi i wsp. 2004). Do chwili obecnej istnieją rozbieżności w ocenie roli PARG w niedokrwieniu mózgu, wynikające z odmiennych danych, uzyskanych na zwierzętach genetycznie modyfikowanych, pozbawionych PARG, i wyników, uzyskanych po zastosowaniu inhibitorów PARG. Może to wynikać ze stosunkowo słabej specyficzności stosowanych inhibitorów dla PARG.

Hipoteza, że hamowanie syntezy PAR zmniejsza wrażliwość tkanki na niedokrwienie, zależy prawdopodobnie od wpływu PAR na profil ekspresji genów (Chiarugi 2002, 2005). PAR wchodzi w interakcje ze specyficzną dla niego domeną w licznych czynnikach transkrypcyjnych, w tym NF-kappaB, p53, AP-1, i w ten

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sposób reguluje ekspresję wielu genów prozapalnych. Inhibicja PARP-1, a więc obniżenie PAR, redukuje ekspresję takich prozapalnych białek, jak CD1lb, ICAM-1, COX2 (Koh i wsp. 2004, Chiarugi 2005). PAR poprzez wpływ na ekspresję genów moduluje i hamuje aktywację mikrogleju, astrocytów, hamuje infiltrację neutrofilii. PARG, podobnie jak PARP-1, poprzez regulację poziomu PAR i regulację transkrypcji, ma istotne znaczenie w procesie różnicowania komórek (Di Meglio i wsp. 2003) oraz w procesie ich obumierania (Affar i wsp. 2001, Ying i wsp. 2001). Badania Ying i wsp. (2000, 2001) wykazały, że inhibicja PARG przez gallotaninę lub nobotanninę powoduje wzrost poziomu PAR, zapobiega przed zależnym od PARP-1 obniżeniem NAD^+ w neuronach i astrocytach. Dane te wskazują, że aktywność PARG zapobiega inhibicji PARP-1 przez autorybozylację i pozwala na podtrzymanie syntezy PAR w neuronach. Wobec powyższego uważa się, że PARG spełnia istotną rolę w utrzymaniu homeostazy PAR i nadmierna aktywacja tego enzymu może być szkodliwa np. w patologii niedokrwiennej mózgu. Tak więc wtedy aktywność enzymu winna być hamowana zgodnie z sugestiami Lu i wsp. (2003). Jednakże do chwili obecnej wyniki badań, dotyczące znaczenia PARG w niedokrwieniu mózgu, są nadal kontrowersyjne, podobnie zresztą, jak wyniki badań dotyczące roli PARP-1 w globalnym niedokrwieniu mózgu.

Wyniki własnych badań wskazują, że PARP odgrywa istotną rolę w obumieraniu neuronów w wyniku krótkiego globalnego niedokrwienia mózgu (Strosznajder i wsp. 2003, 2005b). Stwierdzono, że niedokrwienie mózgu powoduje zaburzenia w uwalnianiu bardzo istotnych wtórnych przekaźników informacji pochodzenia lipidowego, takich jak diacyloglicerol, inozytolofosforany oraz kwas arachidonowy (Strosznajder i wsp. 1999). Te ważne przekaźniki informacji, uwalniane w nadmiarze w niedokrwieniu mózgu, powodują wzrost stężenia wewnątrzkomórkowego wapnia i uwalnianie licznych, wolnych rodników, co może prowadzić do uszkodzenia DNA, aktywacji PARP, a w konsekwencji do zaburzeń funkcji neuronów i astrocytów. Przeprowadzone przez nas badania (Strosznajder i wsp. 2003) wskazują na różną dynamikę zmian aktywności PARP w wyniku zastosowania 3 i 10 min globalnego niedokrwienia mózgu. Zastosowany inhibitor PARP, 3-AB, zapobiegał obumieraniu neuronów w warstwie CA1 hipokampa tylko w przypadku zastosowania 3 min niedokrwienia (Strosznajder i wsp. 2003).

Analiza poli(ADP-rybozylacji) białek wykazuje, że liczne białka, poza samym PARP-em, ulegają ADP-rybozylacji i te zmiany białek mogą leżeć u podstaw zmian patologicznych. PARP powoduje np. zahamowanie dehydrogenazy aldehydu

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3-fosfoglicerynowego, kluczowego enzymu glikolizy (Szabo i Dawson 1998, Du i wsp. 2003), i obniża produkcję ATP w procesie glikolizy oraz metabolizm glukozy w mitochondrialnym cyklu kwasów trójkarboksylowych.

Zaburzenia energetyczne, spowodowane nadmierną aktywnością PARP, mogą być przyczyną nekrotycznej śmierci komórek. Obecnie wiadomo, że PARP poprzez wyczerpanie mitochondrialnej puli βNAD^+ ma być odpowiedzialny za uwalnianie z mitochondriów czynnika indukującego apoptozę (apoptosis inducing factor AIF), który to związek powoduje apoptotyczną śmierć komórek, niezależną od kaspaz a zależną od PARP (Yu i wsp. 2002). Do niedawna uważano, że PARP pełni wyłącznie bierną rolę w apoptozie a jego degradacja jest istotnym wskaźnikiem obumierania komórek na drodze apoptozy, zależnej od kaspaz. PARP jest substratem dla kaspazy 3 i 7. Obecnie wiadomo, że również produkty jego degradacji aktywują apoptozę. PARP 113 kDa jest degradowany najczęściej na dwie części: fragment 89 kDa, zawierający domenę katalityczną, która nie może być aktywowana przez uszkodzenie DNA, oraz 24 kDa fragment, zawierający domenę wiążącą DNA (DBD). Fragment 24 kDa może nieodwracalnie wiązać się z DNA i hamować jego naprawę oraz zapobiegać wyczerpaniu βNAD^+, a tym samym przyspieszać apoptozę (Smulson i wsp. 1998, D'Amours i wsp. 2001). Fragment 89 kDa może natomiast wchodzić w interakcję z nietkniętym PARP-1 i może blokować homodimeryzację PARP-u, konieczną dla aktywności tego enzymu i jego funkcji w naprawie DNA.

Wyniki badań ostatnich lat wskazują na istotną rolę PARP nie tylko w decyzji o przeżyciu lub śmierci komórki, ale także o tym, na jakiej drodze (nekrotycznej czy apoptotycznej) następuje śmierć komórki. Wyjaśnienie roli PARP w krótkim globalnym niedokrwieniu mózgu może pomóc w terapii nagłego zatrzymania krążenia, np. w przypadku zatrzymania akcji serca.

Plaschke i wsp. (2000) obserwowali neuroprotekcyjny efekt inhibitorów PARP-1 w globalnym niedokrwieniu mózgu u szczura. Badania Nagayamy i wsp. (2000) sugerują, że wzrost aktywności PARP-1 w hipokampie szczura po przejściowym globalnym niedokrwieniu mózgu może mieć znaczenie neuroprotekcyjne. Jednakże w stosowanym przez Nagayamę (2000) modelu niedokrwienia mózgu poziom βNAD^+ nie ulegał zmianie. Zahamowanie aktywności PARP obniżało natomiast ilość przeżywających neuronów w hipokampie w 72 godzinie po niedokrwieniu mózgu. Moroni i wsp. (2001) nie obserwowali również protekcyjnego działania inhibitora PARP po 5 min niedokrwieniu mózgu u gerbila. Należy jednak zwrócić uwagę, że w tym przypadku benzamid był podawany

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dootrzewnowo w bardzo wysokiej dawce, 2 godziny przed wywołaniem niedokrwienia. Reasumując, należy stwierdzić, że przyczyna istniejących rozbieżności w opublikowanych wynikach może być związana z różnymi czasami trwania niedokrwienia, jak również dużą zmiennością stosowanych dawek i drogi podania potencjalnych leków.

6. Perspektywy zastosowania inhibitorów PARP-1 w klinice

Rozwój badań nad selektywnymi inhibitorami PARP-1 mającymi jednocześnie niewielkie efekty uboczne, może przynieść korzyści kliniczne nie tylko w badaniach nad niedokrwieniem mózgu czy urazowym uszkodzeniem mózgu i rdzenia kręgowego, ale również w chorobach neurodegeneracyjnych a także w walce z procesami nowotworzenia. Intensywne badania nad wyjaśnieniem mechanizmów przeżycia i śmierci komórek nerwowych stwarzają szansę na wprowadzenie skutecznej terapii w niedalekiej przyszłości. Ostatnio w artykule przeglądowym Graziani i Szabo (2005) przedstawili stan obecnej wiedzy na temat możliwości i perspektyw zastosowania inhibitorów PARP w leczeniu nowotworów, ostrych schorzeń sercowo-naczyniowych oraz ostrych i przewlekłych chorób neurologicznych. Autorzy podkreślają, że pomimo intensywnych badań nad PARP-em w ostatnim dziesięcioleciu i licznych wskazań celowego farmakologicznego zastosowania inhibitorów PARP-1, niestety, nie ma do tej pory żadnego związku, który byłby już stosowany w klinice. Próby kliniczne wybranych inhibitorów PARP-1 rozpoczęły się w 2003 roku i najbliższe lata przyniosą dopiero odpowiedź na pytanie odnośnie ich skuteczności.

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III. CEL BADAŃ ROZPRAWY HABILITACYJNEJ

Celem badań było scharakteryzowanie enzymu jądrowego polimerazy poli(ADP-rybozy) (PARP-1) w różnych częściach mózgu dojrzałego i starczego, w warunkach stresu oksydacyjnego wywołanego działaniem peptydów amyloidu beta, oraz ocena udziału PARP-1 w procesach przeżycia i obumierania komórek w patologii niedokrwiennej mózgu.

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V. PODSUMOWANIE I WNIOSKI SFORMUŁOWANE NA PODSTAWIE WYNIKÓW BADAŃ ZAŁĄCZONYCH PRAC

Opisano po raz pierwszy w literaturze zmiany w aktywności polimerazy poli(ADP-rybozy) (PARP-1) w hipokampie, korze, prążkowiu i móżdżku u zwierząt dorosłych i starych. Stwierdzony wzrost aktywności PARP-1 we wszystkich badanych strukturach mózgu zwierząt starych może być wyrazem zwiększonego uszkodzenia DNA i wskazywać na udział tego enzymu w procesach naprawy. Brak zmian w poziomie białka dla PARP-1 w mózgach starczych świadczyć może o posttranslacyjnej, kowalencyjnej modyfikacji enzymu.

Stwierdzony brak odpowiedzi PARP-1 w hipokampie zwierząt starych na pobudzenie receptorów glutaminianergicznych typu NMDA i na działanie peptydów amyloidu beta (Aβ) oraz na wywołany jonami żelaza stres oksydacyjny wskazuje na uruchomienie mechanizmów zabezpieczających hipokamp starczy przed nadmierną aktywacją PARP-1.

Peptydy amyloidu beta w hipokampie zwierząt dorosłych powodują wzrost poziomu wolnych rodników i uszkodzenie DNA, co w konsekwencji doprowadza do aktywacji PARP-1. Stwierdzone natomiast obniżenie zależnego od receptora cholinergicznego uwalniania lipidowych przekaźników informacji, w tym inozytolo(1,3,5)trisfosforanu (IP$_3$), przez peptydy Aβ może zaburzać proces przekaźnictwa sygnału regulowanego przez IP$_3$/Ca^{2+} do PARP-1.

Po raz pierwszy w literaturze opisana została dynamika zmian aktywności PARP-1 w różnych czasach przywrócenia krążenia po 3 i 10 min globalnym niedokrwieniu mózgu. Zahamowanie aktywności PARP-1 bezpośrednio po krótkim epizodzie niedokrwiennym znamiennie chroniło neurony w warstwie CA1 hipokampa przed obumieraniem. W przypadku 10 min niedokrwienia inhibicja PARP nie zapobiegała obumieraniu neuronów w warstwie CA1 hipokampa. Uzyskane wyniki wskazują na korzystne neuroprotekcyjne działanie inhibitora PARP-1 w krótkim globalnym niedokrwieniu mózgu.

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W mechanizmie protekcyjnego działania inhibitora PARP-1 w globalnym niedokrwieniu mózgu istotne znaczenie miał jego ochronny wpływ nie tylko na neurony ale również na komórki glejowe (astrocyty) oraz perycyty.

3-aminobenzamid zmniejszał obrzęk cytotoksyczny, miał korzystny wpływ na zachowanie prawidłowej ultrastruktury organelli wewnątrzkomórkowych, w szczególności mitochondriów.

Ponadto stwierdzono, że antagonista receptorów beta adrenergicznych, karwedilol, zmniejszał w istotny sposób obumieranie neuronów warstwy CA1 hipokampa po 5 min globalnym niedokrwieniu mózgu. Karwedilol zapobiegał aktywacji PARP-1 oraz obniżeniu stężenia βNAD^+, co mogło mieć istotny wpływ na uzyskaną neuroprotekcję.

Wyniki przedstawionych badań wyraźnie wskazują, że zahamowanie aktywności polimerazy poli(ADP-rybozy) bezpośrednio po zaistnieniu krótkich epizodów niedokrwiennych może być skuteczną metodą w zapobieganiu obumierania neuronów mózgu.

dra. W pracy wysłanej do publikacji (manuskrypt pracy w załączeniu) stwierdzono,
w 4 dniu przywrócenia krążenia po 3 min niedokrwieniu AIF uwalniany jest z
itochondriów do cytoplazmy i translokowany do jądra. Podanie dożylne inhibitora
ARP, 3-aminobenzamidu, bezpośrednio po 3 min niedokrwieniu mózgu zatrzymuje
anslokację AIF do jądra. AIF był głównie widoczny w niezmienionych
rukturalnie mitochondriach i aparacie Golgiego. Ponadto, po podaniu 3-AB
adekspresja białka Bcl-2 była widoczna w błonach mitochondrialnych, w siateczce
ódplazmatycznej, aparacie Golgiego, błonach jądrowych, jak również w
toplazmie i jądrze. Wyniki te wskazują, że inhibicja aktywności PARP może mieć
rzystny efekt na neurony hipokampa poprzez wyraźny wzrost ekspresji białka Bcl-
oraz zmniejszenie uwalniania AIF z mitochondriów i zahamowanie jego
anslokacji do jądra. Badania mają być kontynuowane z uwzględnieniem roli innych
ałek pro- i antyapoptotycznych oraz z uwzględnieniem udziału czynników
anskrypcyjnych, w tym białka p53 i NF-kappaB, w procesach przeżycia i śmierci
mórek, w patologii niedokrwiennej mózgu.

VII. KOPIE PUBLIKACJI WYMIENIONYCH W WYKAZIE (IV)

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Amyloid β Peptide 25–35 Modulates Hydrolysis of Phosphoinositides by Membrane Phospholipase(s) C of Adult Brain Cortex

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Abstract

Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in signal transduction. A subset of muscarinic cholinergic receptors are linked to G-proteins that activate phospholipase C. Cholinergic pathways are important in learning and memory, and deficits in cholinergic transmission have been implicated in Alzheimer's disease (AD). AD is also associated with increased β-amyloid plaques.

In the present study, we have investigated the effect of the amyloid β (Aβ)synthetic peptide homologous to residue 25–35 of Aβ in nonaggregated and aggregated forms on the degradation of inositol phospholipids. Synaptic plasma membranes (SPM) and the cytosolic fraction from rat brain cortex served as a source of enzymes. The studies were carried out with radioactive inositol phospholipids in the presence of endogenous and 2 mM $CaCl_2$. The enzyme(s) activity was evaluated by determination of the product formation of [³H]inositol-1-phosphate (IP_1) or [³H]inositol-1,4,5-trisphosphate (IP_3). Results show that the PI-PLC activity was significantly higher in cytosol compared to SPM, and this enzyme was stimulated by 2 mM $CaCl_2$, but not by GTPγS or carbachol, a cholinergic receptor agonist. Activity of the SPM-bound PIP_2-PLC was similar to that in cytosol and was not activated by 2 mM $CaCl_2$. The SPM PIP_2-PLC was significantly stimulated by GTPγS together with the cholinergic agonist, carbachol. Freshwater-soluble Aβ 25–35 activated PI-PLC in SPM markedly by two- to threefold, but this effect was absent in the presence of 2 mM $CaCl_2$. Moreover, Aβ 25–35 had no effect on basal PIP_2-PLC activity and cytosolic PI-PLC and PIP_2-PLC. The aggregated form of Aβ 25–35 significantly inhibited PIP_2-PLC only in the presence of endogenous $CaCl_2$. It also inhibited the carbachol and GTP(γ)S-stimulated PIP_2-PLC. Our findings show that depending on the aggregation state and Ca^{2+} concentration, Aβ modulates phosphoinositide degradation differently and exclusively in brain synaptic plasma membranes. Our data suggested that aggregated Aβ peptide may be responsible for the significant impairment of phosphoinositide signaling found in brain membranes during AD.

Index Entries: Amyloid β peptide; phospholipase C; phosphoinositides; synaptic plasma membrane.

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Preparation of Brain Cortex Homogenate, Synaptic Plasma Membrane, and Cytosol

Adult male Wistar rats, 4 mo old, were used for the experiments. Animals were decapitated, and the brains were rapidly removed. A 10% homogenate was obtained by homogenization of dissected brain cortex and hippocampus in a Dounce-type glass homogenizer in 0.32 M sucrose with 10 mM Tris-HCl (pH 7.4). The homogenate (10%, w/v) was centrifuged for 3 min at 1100g. The pellet (P_1) containing the nuclear fraction and nondisrupted cells was discarded. The resulting supernatant (S_1) was centrifuged for 10 min at 17,000g to yield a crude synaptosomal-mitochondrial fraction (P_2). Subsequently, the P_2 pellet was subjected to hypotonic shock with 1 mM Tris-HCl (pH 7.0) by homogenization and was centrifuged at 1000g for 10 min. The pellet was again subjected to hypotonic shock and centrifuged at the same conditions. Finally, the combined supernatants, after adjustment to 0.32 M sucrose, were centrifuged at 48,000g for 20 min to obtain the synaptic plasma membranes (SPM). Cytosol was obtained by centrifugation of the 17,000g supernatant at 104,000g for 60 min.

Assays for PLC Activity

The enzyme activity was assayed by measuring the formation of [^{3}H]inositol phosphate(s) released from labeled substrate. PIP_2 (10 nmol) (2.5–2.8 × 10^4 dpm) or 20 nmol PI (2.0 × 10^4 dpm) was added/incubation vial, and organic solvent was evaporated under nitrogen. After addition of 20 mM Tris-HCl (pH 6.6) for determination of PLC acting on PIP_2 or 20 mM Tris-HCl (pH 7.8) in the case of PI, and 0.1% sodium deoxycholate, each tube was vigorously vortexed for 1 min. The assay systems also contained 10 mM LiCl, 0–2.0 mM $CaCl_2$, 2 or 10 mM ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetra-acetic acid (EGTA), and 50 μg protein in a final volume of 200 μL. Mixtures were incubated for 30 min at 37°C in the case of PI-PLC and 15 min for PIP_2-PLC. The reactions were stopped with 1 mL chloroform/methanol/concentrated HCl (100:100:0.6 by volume), and then 0.3 mL H_2O and 1 mL of chloroform were added. After a 15-min incubation and centrifugation at 1500g for 5 min, the aqueous upper phase was transferred to separate tubes, and then a drop of NH_4OH was added. Samples were mixed, centrifuged at 1500g for 5 min, and a 0.4-mL portion of the aqueous phase was then mixed with 8 mL of Bray's scintillation fluid for determination of radioactivity using LKB Wallach 1409 counter.

Statistics

Statistical analysis was conducted using analysis of variance (ANOVA) and Newman-Keuls test post-hoc. Significance was defined as $p < 0.05$.

Results

The effects of Aβ peptides on the activity of the PLC acting on PI and PIP_2 were determined in the presence of endogenous calcium concentrations in SPM and cytosol fractions from rat brain cortex using exogenous inositol phospholipids. The results confirmed our previous observations, and indicated that cytosolic PI-PLC has a fourfold higher activity compared to the SPM-bound PI-PLC and that enzyme in the cytosolic fraction was markedly stimulated by 2 mM $CaCl_2$ (F [5,12] = 40, $p < 0.0001$) (Fig. 1). The specific activity of the SPM-bound PIP_2-PLC was 10-fold higher than that for PI-PLC, but this enzyme activity was similar in the cytosol and SPM. Calcium ions at millimolar concentration had no additional stimulatory effect on the PIP_2-PLC (Table 1). The neurotoxic peptide, Aβ 25–35, markedly influenced only the activity of SPM-bound PI and PIP_2-PLC, but it had no effect on cytosolic PI-PLC and PIP_2-PLC activities (data not shown). Hydrolysis of PI by PLC was unchanged by stimulation of cholinergic receptors in the presence of GTPγS and endogenous or 2 mM $CaCl_2$.

For characterization of the SPM-bound PLC, the activity of the enzyme was measured without or with Ca^{2+}, carbachol, and GTPγS. There are two different pathways for phosphoinositide degradation in rat brain cortex, namely Ca^{2+}-dependent PI-PLC and G-protein-regulated, receptor-mediated PIP_2-PLC (Fig. 1 and Table 1). Subsequently, several experiments were carried out to determine the role of Aβ 25–35 in the modulation of PI and PIP_2-PLC activity. For the determination of the effect of Aβ peptide on PI-PLC and PIP_2-PLC, the final con-

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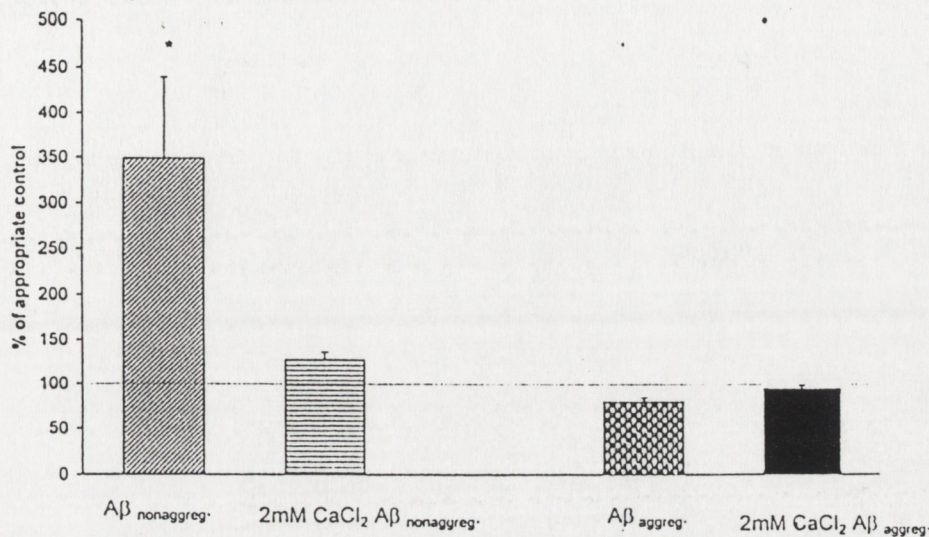
Fig. 2. Differences in effects of Aβ 25–35 on synaptic plasma membrane PI-PLC depending on peptide aggre-
tion and calcium concentration. The data were evaluated by one-way ANOVA (F [4,10] = 22.82, $p < 0.0001$)
atistical significance was determined by a post-hoc Newman-Keuls test. Values are means ± SD from 3 exper-
ents carried out in triplicate. Aβ 25–35 was dissolved in double distilled water at 2.5 mM and added into the
cubation vial at a final concentration of 25 µM. The aggregated Aβ was obtained by incubation of the 2.5 mM
solution at room temperature for 7 d. *$p < 0.0005$ vs the control value of PI-PLC activity in the absence of
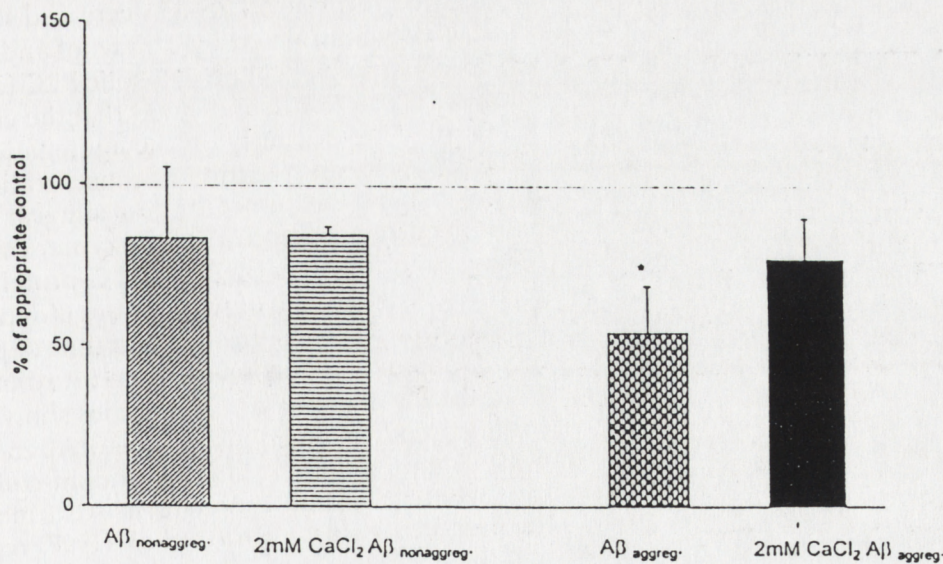ogenous CaCl₂ and Aβ.

Fig. 3. Aggregated Aβ 25–35 significantly inhibits PIP₂-PLC activity. The data were evaluated by one-way ANOVA
[4,10] = 4.89, $p < 0.05$). Statistical significance was determined by a post-hoc Newman-Keuls test. Values are
ans ± SD from 3 experiments carried out in triplicate. *$p < 0.05$ vs the control value of PIP₂-PLC activity in the
sence of exogenous CaCl₂ and Aβ.

phospholipids (Seelig et al., 1995). This type of Aβ interaction with phospholipid may play an important role in modulation of the activity of membrane-bound enzymes.

Labeled phosphoinositides as substrates and membrane fractions as a source of enzyme are satisfactory for the measurement of G-protein-mediated and receptor-induced phosphoinositide hydrolysis (Strosznajder and Strosznajder, 1989; Crews et al., 1994; Jope, 1996; Jope et al., 1997). This method was used to evaluate the role of Aβ on degradation of phosphoinositides and to compare our data with those obtained with AD membranes (Crews et al., 1994; Jope, 1996; Jope et al., 1997). Our results are consistent with data obtained by Kelly et al. (1996), suggesting that aggregated amyloid peptides are important compounds that may be responsible for the disturbances of phosphoinositide signaling in AD brain previously observed (Crews et al., 1994; Jope, 1996). In evaluating the role of Aβ, it is important to remember that Aβ deposition is a necessary, but perhaps not sufficient factor for the disturbance of neurotransmission and signal transduction processes and pathogenesis in AD (Selkoe, 1994; Soto et al., 1997). Our data differ from those who reported that Aβ 25–35 stimulates activities of phospholipase A, C, and D (Singh et al., 1995, 1996, 1997). Abnormalities of the neuroblastoma cell line LA-N-2 (Singh et al., 1995, 1996, 1997) and different assay conditions may account for the differences.

In LA-N-2 neuroblastoma cells, Singh et al. (1997) observed that the stimulation of PLC by Aβ is independent of Ca^{2+} and protein kinases, but appeared to be pertussis toxin-sensitive. Using brain SPM, we have found that Aβ in a nonaggregated form stimulates PLC at low endogenous Ca^{2+}, but Aβ has no effect at 2 mM $CaCl_2$. We have also observed that Aβ 25–35 activates Ca^{2+} influx into brain cortex synaptoneurosomes through voltage-operated Ca^{2+} channels at low KCl concentration, but has no effect on the maximal rise in $[Ca^{2+}]$ induced by 75 mM KCl (Samochocki et al., 1998). Our data are consistent with those reported by Hartmann et al. (1996).

Our data demonstrated that the aggregated Aβ significantly decreased the activities of SPM-bound PIP_2-PLC. This peptide has a greater inhibitory effect in the presence of endogenous $CaCl_2$ and decreased PIP_2-PLC activity by about 40%. Aβ 25–35 also inhibits the PIP_2-PLC activity stimulated by GTPγS and carbachol by about 11 and 35%, respectively, and decreases by about 17% the PIP_2-PLC activity regulated by GTPγS and carbachol together.

The basal activity of PIP_2-PLC was stimulated by carbachol and GTPγS. The stimulation by carbachol and GTPγS in the absence of Aβ was about 30%. In the presence of Aβ, the stimulation was about 90% in spite of the significantly lower PIP_2-PLC activity observed in the presence of Aβ in all investigated conditions. Aβ exerted a similar effect on carbachol and GTPγS-regulated PIP_2-PLC activity in the presence of endogenous and 2 mM $CaCl_2$. Our results suggest that Aβ, through alteration of the interaction of SPM-bound PLC with Ca^{2+} and G-protein, significantly influences the pathways for signal transduction in the brain.

Acknowledgment

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Alteration of Phosphoinositide Degradation by Cytosolic and Membrane-Bound Phospholipases after Forebrain Ischemia - Reperfussion in Gerbil: Effects of Amyloid Beta Peptide

Joanna Strosznajder,[1] Agata Zambrzycka,[1] Maria D. Kacprzak,[1] Dorota Kopczuk,[1] and Robert P. Strosznajder[2]

The reperfusion of previously ischemic brain is associated with exacerbation of cellular injury. Reperfusion occasionally potentates release of intracellular enzymes, influx of Ca^{2+}, breakdown of membrane phospholipids, accumulation of amyloid precursor protein or amyloid β-(like) proteins, and apolipoprotein E. In this study, the effect of reperfusion injury on the activity of cerebral cortex enzymes acting on phosphatidyl [^{3}H] inositol (PI) and [^{14}C-arachidonoyl] PI was investigated. Moreover the effect of amyloid β25–35 on PI degradation by phospholipase(s) of normoxic brain and subjected to ichemia-reperfussion injury was determined. Brain ischemia in gerbils (*Meriones unguiculatus*) was induced by ligation of both common carotid arteries for 5 min and then brains were perfused for 15 min, 2 h and 7 days. Statistically significant activation of enzyme(s) involved in phosphatidylinositol degradation in gerbils subjected to ischemia-reperfusion injury was observed. Nearly all gerbils showed a higher activity of cytosolic PI phospholipase C (PLC) at 15 min after ischemia. Concomitantly, the significant enhancement of the level of DAG and AA radioactivity at this short reperfusion time confirmed the active PI degradation by phospholipase(s) in cerebral cortex and hippocampus. After a prolonged reperfusion time of 7 days after ischemia, both cytosolic and membrane-bound forms of PI-PLC were activated. The question arises if alteration of membranes by the degradation of phospholipids occurring after an ischemic episode potentates the effect of Aβ on membrane-bound enzymes. A neurotoxic fragment of amyloid, Aβ 25–35, incubated in the presence of endogenous Ca^{2+}, increased significantly the PI-PLC activity of normoxic brain. In its non-aggregated form, Aβ 25–35 activates PI-PLC but in the aggregated form the enzymatic activity decreased. Thus, Aβ 25–35 exerts a similar effect on the membrane-bound PI-PLC from normoxic brain or subjected to ischemia reperfussion injury. We conclude that the degradation of phosphatidylinositol by cytosolic phosphoinositide-phospholipase C may contribute to the pathophysiology of delayed neuronal death following cerebral ischemia. Thus, a specific inhibitor of this enzyme(s) may offer therapeutic strategies to protect the brain from damage triggered by ischemia. Ischemia-reperfusion injury had no effect on Aβ-evoked alterations of synaptic plasma membrane-bound PI-PLC.

INTRODUCTION

The reperfusion of previously ischemic brain is associated with exacerbation of cellular injury as judged

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Preparation of Cerebral Cortex Homogenate. The animals were decapitated and the brains were removed rapidly. The 10% homogenate was obtained by homogenization of dissected cerebral cortex and hippocampus in 0.32 M sucrose with 10 mM Tris-HCl buffer pH 7.4 in a Dounce-type glass homogenizer.

Preparation of Synaptic Plasma Membrane and Cytosol. The subcellular fractions were isolated from dissected brain hemispheres homogenized in a Dounce-type glass homogenizer in ice-cold isolation medium, containing 0.32 M sucrose and 10 mM Tris-HCl buffer pH 7.4. The homogenate (10%, w/v) was centrifuged for 3 min at 1,100 g. The pellet (P_1) containing the nuclear fraction and undisrupted cells was discarded. The resulting supernatant (S_1) was centrifuged for 10 min at 17,000 g to yield a crude synaptosomal-mitochondrial fraction (P_2). Subsequently, the pellet was subjected to hypotonic shock with 1 mM Tris-HCl buffer pH 7.0 by homogenization and then was centrifuged at 1,000 g for 10 min. The pellet was again subjected to hypotonic shock and centrifuged under the same conditions. Finally, the combined supernatants, after adjustment to 0.32 M sucrose, were centrifuged at 48,000 g for 20 min to obtain the synaptic plasma membranes (SPM). The supernatant (S_2) from the 17000 g centrifugation was then centrifuged at 104,000 g for 60 min to obtain the supernatant, the cellular cytosol.

Assays for Phospholinase C Activity. The activity of phospholipase C acting on PI was assayed by measuring the formation of the radioactive water-soluble metabolite, inositol monophosphate, from labeled substrate. Exogenous substrate, 20 nmol [^{3}H-inositol]PI, was added to each incubation vial, and the organic solvent was evaporated under nitrogen. After addition of 20 mM Tris-HCl buffer, pH 7.8, and 0.1% sodium deoxycholate, each tube was vigorously vortexed for 1 min. In addition, the assay system contains 10 mM LiCl and about 50 µg protein in a final volume of 200 µl. Mixtures were incubated for 30 min at 37°C. The reactions were stopped with 1.0 ml chloroform/methanol/concentrated HCl 100:100:0.6 (by vol.), then 1.0 ml of chloroform and 0.3 ml H_2O were added. A 0.5 ml portion of the aqueous phase was mixed with 8 ml of Bray's scintillation fluid for determination of radioactivity. The remainder of the aqueous phase was used for the separation of radioactive water-soluble inositol metabolites.

Effect of Amyloid β-Peptide on PI Phospholipase C Activity. Aβ 25–35 was dissolved in sterile distilled water (vehicle) at a concentration of 2.5 mM and was used directly for the experiments or it was incubated at 2.5 mM concentration at room temperature for 7 to 9 days. For the determination of the effect of Aβ peptide on PI-PLC, the final concentration of Aβ 25–35 was 25 µM. The presence of aggregated Aβ was assessed by light microscopy. The fresh Aβ peptide dissolved in double-distilled H_2O was evaluated with circular dichroism (CD) for the percentage distribution of structural forms of Aβ.

Circular Dichroism Spectroscopy. Results are expressed in terms of the mean residue ellipticity of the Aβ 25–35 in units of 10^{-3} deg cm²/decimol (dmol). All spectra were corrected by subtracting the buffer baseline. CD measurements were carried out on a CD 62DS spectrometer equipped with a computerized data processor. Peptide samples were solubilized as described above 24 h before analysis and diluted to 25 µM in double-distilled H_2O. Samples were read at room temperature in a 0.5 cm pathlength quartz cell. Measurements were made over a 190 to 250 nm wavelength range, taken at 5 nm increments.

Separation of Water Soluble Inositol Metabolites. The water-soluble inositol metabolites were separated using a small column (0.5 × 7.0 cm) containing 0.4 g Dowex AG 1 × 8 (200–400 mesh). Free inositol, glycerophosphoinositol, and inositol monophosphate were eluted sequentially according to Berridge et al. (1983).

Assay of [^{14}C]Arachidonic Acid Release from Labeled Substrates. The entire procedure was carried out in the presence of 200 µg of protein, using 25 nmol of [^{14}C-AA] PI (3 × 10⁴ dpm), 0.1% deoxycholate, 20 mM Tris-HCl pH 7.8 in 200 µl of incubation mixture. The labeled AA, DAG and other lipids were extracted (7).

Separation of Lipids. Free fatty acids (FFA) and diacylglycerol (DAG) were separated by TLC using chloroform/acetone 96/4 (v/v) or chloroform/methanol/4M ammonium hydroxide 9/7/2 (by vol). The lipid areas visualized with iodine vapor and corresponding to lipid standards were scraped from TLC plates into the scintillation vials. Radioactivity was counted using 10 ml of Bray's scintillation fluid in a LKB Wallac 1409 counter.

Statistics. Differences in responses were assessed for significance by paired t-test. Differences were considered statistically significant for $p < 0.05$.

RESULTS

The degradation of phosphatidyl [^{3}H-inositol] by the cytosolic and synaptic plasma membrane bound phospholipase C (PLC) of cerebral cortex and hippocampus (taken together) was evaluated. About 90% of animals submitted to ischemia-reperfusion injury had a significant enhancement of cytosolic PLC activity at 15 min and 7 days after ischemic insult. In addition, independent experiments on the dependency of PI-PLC activity on time, protein concentration, and substrate concentration confirmed the higher activity of cytosolic PI-PLC at 15 min after ischemia (data not shown). The activity of PI-PLC in the synaptic plasma membrane fraction was also enhanced by about 34% at 7 days after a short period of forebrain ischemia, but no significant stimulation was seen at earlier times after ischemia (Table I). These results were obtained using 12 to 16 gerbils for each experimental group (control and each reperfusion time). In one experiment (2 animals) the PI-PLC activity was not changed at 15 min after ischemia. In 8 experiments carried out on 16 gerbils, the ischemia reperfusion had no effect in only one, so this experiment was excluded from the evaluation.

The main water-soluble product of phosphatidyl[^{3}H]inositol degradation was inositol monophosphate. The low level of radioactivity in glycerophosphoinositol was slightly but insignificantly increased (data not shown).

Another substrate, [^{14}C-arachidonoyl] phosphatidylinositol, was used to evaluate the formation of lipid mediators. A significant enhancement of the level of labeled arachidonic acid (AA) and diacylglycerol (DAG) was found at 15 min after ischemia in cerebral cortex and hippocampus (Tables II and III), but not at 2 h or 7 days. The results 15 min after

Table III. Degradation of [¹⁴C-Arachidonoyl] Phosphatidylinositol by Membrane-Bound and Cytosolic Enzyme(s) of Hippocampus after Ischemia-Reperfusion Injury

| Reperfusion time | % of control | | | |
| | SPM | | Cytosol | |
	AA	DAG	AA	DAG
15 min	98.6 ± 6.3	105.8 ± 26.3	$117.8 \pm 13.0*$	$258.0 \pm 164.0*$
2 h	98.1 ± 6.5	107.1 ± 22.0	84.3 ± 13.0	131.7 ± 82.0
7 days	110.9 ± 12.0	122.8 ± 26.0	101.3 ± 43.1	107.6 ± 24.9

For each experiment 2 animals were used for the preparation of synaptic plasma membranes (SPM) and cytosol. In most cases, 5 experiments were carried out. The radioactivity in the AA and DAG was determined as described in Materials and Methods and results are presented as percentages of control (sham operated animals). The control value for AA and DAG released from PI by SPM enzyme(s) was in the range of $0,25 \pm 0,03$ and $0,59 \pm 0,05$ nmol/mg protein/min respectively. The control value for AA and DAG released from PI by cytosolic enzyme(s) was in the range $0,41 \pm 0,06$ and $1,47 \pm 0,16$ nmol/mg protein/min respectively. Degradation of [¹⁴CAA]—PI was determined in the presence of endogenous $CaCl_2$ concentration as described in Experimental Procedure. The values are means $\pm$ SD from 3 to 5 experiments carried out in triplicate. Statistical significance was evaluated by Student test-t, *$p < 0.05$ versus the value of control.

In this study, during reperfusion, arachidonic acid was significantly and specifically released by cytosolic enzyme(s), probably by PLC and DAG lipase, we are not able to exclude the involvement of PI-PLA_2. Clemens (15) reported an enhancement of cytosolic $cPLA_2$ activity in the brain following transient global ischemia that may precede the onset of neuro-nal death. Yoshihara (56) and Clark (14) have isolated the cytosolic PLA_2 that belongs to the high molecular weight type and its activity is regulated by a translocation mechanism. We have shown that activation of NMDA receptors is responsible for the enhancement of NO synthase activity during reperfusion (11). Moreover, we suggested that the NO-dependent inhibition of AA incorporation into membrane lipids might also be responsible for increases of the AA level in brain during reperfusion 47,44). Inhibition of
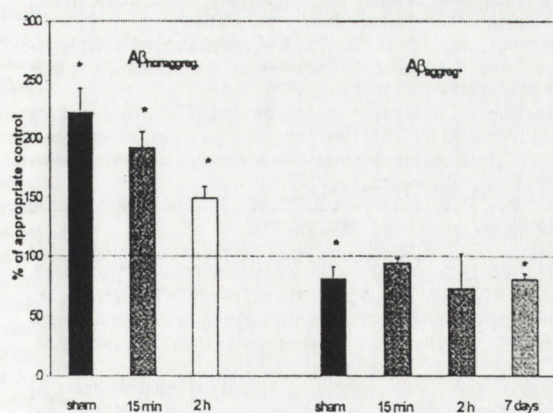

Fig. 1. Effects of soluble and aggregated Aβ (25–35) on the activity of phospholipase C (PLC) in cerebral cortex homogenate from animals subjected to brain ischemia—reperfussion injury in the presence of endogenous $CaCl_2$. Aβ (25–35) was dissolved in double-distilled water at 2.5 mM and added into the incubation vial at a final concentration of 25 μM. Aggregated Aβ was obtained by incubation of the 2.5 mM Aβ solution at room temperature for 7 to 9 days. Values are means $\pm$ SD from three experiments carried out in triplicate.
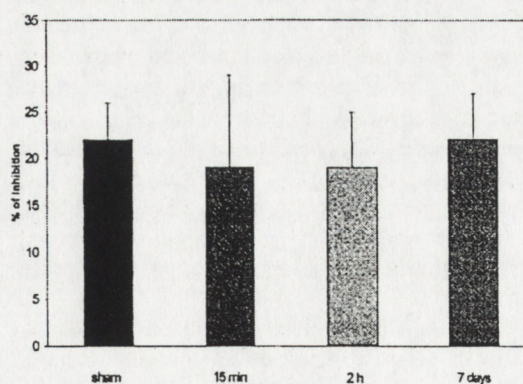

Fig. 2. Percentage inhibition evoked by aggregated Aβ 25–35 on the activity of synaptic plasma membrane phospholipase C (PI-PLC) from the brain subject to ischemia—reperfussion injury. The details of the procedure were described in Experimental Procedure. Values are means $\pm$ SD from three experiments carried out in triplicate.

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Effect of amyloid beta peptide on poly(ADP-ribose) polymerase activity in adult and aged rat hippocampus[*]

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It is suggested that the fibrillar amyloid beta peptide (Aβ) in brain plays a direct role in neurodegeneration in Alzheimer's disease, probably through activation of reactive oxygen species formation. Free radicals and numerous neurotoxins elicit DNA damage that subsequently activates poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30). In this study the effect of neurotoxic fragment (25–35) of full length Aβ peptide on PARP activity in adult and aged rat hippocampus was investigated. In adult (4 month old) rat hippocampus the Aβ 25–35 peptide significantly enhanced PARP activity by about 80% but had no effect on PARP activity in cerebral cortex and in hippocampus from aged (24–27 month old) rats. The effect of Aβ peptide was reduced by half by the nitric oxide synthase inhibitor N-nitro-L-arginine. Stimulation of glutamate receptor(s) itself enhanced PARP activity by about 80% in adult hippocampus. However, Aβ 25–35 did not exert any additional stimulatory effect. These results indicate that Aβ, through NO and probably other free radicals, induces activation of DNA bound PARP activity exclusively in adult but not in aged hippocampus.

The neuronal deposition of amyloid beta peptides (Aβ) and neurofibrillary tangle formation are characteristic pathological features of Alzheimer's disease (AD). However, a fundamental question is whether the deposition of amyloid β peptides in brain plays a direct role in neurodegeneration and cell death.

The hippocampal neurones are particularly sensitive to various types of neurotoxic agents including Aβ peptides. Moreover, neuronal degeneration in the hippocampus may contribute to the memory deficit observed in old people with dementia and AD. Alterations of membrane during aging and induced forma-

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Abbreviations: Aβ, amyloid beta protein; AD, Alzheimer disease; PARP, poly(ADP-ribose) polymerase; TBARS, thiobarbituric acid reactive substances; NNLA, N-nitro-L-arginine; NMDA, N-methyl-D-aspartic acid; p-APMSF, 4-amidinophenylmethane-sulfonyl fluoride.

cubated for 20 min or 60 min, in the presence of 2 mM $CaCl_2$ with either the non-aggregated or aggregated form of Aβ peptide 25–35 over the concentration range of 1–25 μM. After incubation the slices were homogenised and used for determination of PARP activity or of thiobarbituric acid reactive substances (TBARS).

Determination of PARP activity. The PARP activity was determined using [*adenine*-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 μl contained: 200 μM [^{14}C]NAD 2 × 10^5 d.p.m., 100 mM Tris/HCl buffer, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 μM 4-amidinophenyl-methane-sulfonyl fluoride (p-APMSF) and 200 μg of protein. The mixture was incubated for 5 min at 37°C, then the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid. Precipitates were collected on Whatman GF/B filters and washed 3 times with the 5% acid. The radioactivity was determined using LKB Wallac 1409 scintillation counter.

Determination of thiobarbituric acid reactive substances. Thiobarbituric acid reactive substances including malondialdehyde, the last product of lipid peroxidation, were determined according to Asakawa & Matsushita [17]. The hippocampal slices were preincubated for 90 min at 37°C and then were incubated for 20 or 60 min with or without 25 μM Aβ. Then the slices were homogenized in ice cold 10 mM Tris/HCl buffer, pH 7.4. The homogenates were resuspended in 10 mM Tris/HCl buffer, pH 7.4, at protein concentrations of approximately 0.5 mg/ml and were incubated for 5 min. After incubation 1 ml of 30% trichloroacetic acid, 0.1 ml of 5 M HCl and 0.75% thiobarbituric acid were added. The tubes were capped and the mixture was heated at 100°C for 15 min in a boiling water bath. After centrifugation the absorbance of the supernatant was determined at 535 nm against the reagent blank.

The research project was accepted by the Institutional Ethics Committee.

RESULTS

The agonist of glutamate receptor (NMDA) at 100 μM concentration enhanced PARP activity by about 80% in adult rat hippocampus but not in aged hippocampus and cerebral cortex (Fig. 1). This NMDA-evoked stimulation of PARP activity was decreased by the inhibitor of NO synthase, *N*-nitro-L-arginine (NNLA) (Fig. 1). NMDA at lower concentrations (1 μM or 10 μM) had an insignificant stimulatory effect on PARP activity. In the experimental condition, activation of glutamatergic receptor by NMDA (100 μM) did not stimulate lipid peroxidation processes determined as TBARS (not shown). For the determination of the effect of amyloid beta peptide on PARP activity, the neurotoxic Aβ fragment 25–35 at 25 μM final concentration was used. The fresh Aβ analyzed by CD spectroscopy had mainly a random coil structure. The aggregated form of the peptide had a beta sheet structure. Aβ peptide 25–35, exclusively in the aggregated form, significantly stimulated PARP activity in hippocampus slices from adult rats (4 months old) (Fig. 2), while free radical dependent lipid peroxidation determined as TBARS was unchanged (Table 1). In cerebral cortex Aβ had no effect on PARP activity. The amyloid beta peptide evoked PARP stimula-

Table 1. Effect of Aβ peptide 25–35 on TBARS concentration in rat hippocampus.

Hippocampal slices from 4 month old rats were preincubated for 90 min at 37°C and then incubated for 20 or 60 min with or without 25 μM Aβ peptide 25–35. TBARS were determined as described in Material and Methods. The results are mean ± SEM from 3 experiments carried out in triplicate.

Experimental condition		TBARS
Time (min)	Addition of Aβ	nmol/mg protein
0	–	0.83 ± 0.08
20	–	3.04 ± 0.26
20	+	2.59 ± 0.14
60	–	2.76 ± 0.18
60	+	2.77 ± 0.28

tion in adult hippocampus was significantly decreased by NNLA (Fig. 2). In hippocampal slices from aged brain (24–27 month old rats), the Aβ peptide 25–35 had no effect on PARP activity (Fig. 2) and did not exert any additional stimulatory effect on the NMDA receptor which enhanced PARP activity in adult hippocampus (not shown).

DISCUSSION

Our results indicate that the glutamate receptor agonist, NMDA enhanced PARP activity in hippocampal slices from adult rats. However, NMDA had no effect on PARP activity in brain cortex and in aged hippocampus. One of the reasons for these differences could be a lower density of glutamate receptor(s) in cerebral cortex and in aged hippocampus as compared to adult hippocampus. It is possible that the NMDA evoked PARP stimulation is connected with DNA damage, that can not be detected by the currently available methods [18]. However, extensive stimulation of NMDA may induce detectable DNA damage, neurodegeneration and cell death. The role of excessive stimulation of NMDA receptor in cerebral ischemia is well known and documented [19, 20]. Nitric oxide synthase activity is elevated after NMDA receptor stimulation [21, 22]. Liberation of NO$^\bullet$ and ONOO$^-$ can lead to the impairment of membrane phospholipids, mitochondrial proteins and DNA [23–25]. It has been shown that DNA damage after ischemic insults may activate PARP [18, 26]. Zhang *et al.* [27] found that PARP inhibitors blocked the NMDA and NO mediated neurotoxicity. Moreover, there are also suggestions that neurodegeneration evoked by amyloid beta peptide may depend on its influence on glutamate receptors [28, 29]. Our data showed that the neurotoxic amyloid beta fragment 25–35 enhanced PARP activity in adult hippocampus. Significantly lower PARP activity observed in aged hippocampus [30] may be responsible for the lack of the effect of Aβ (25–35) on this enzyme. It is worth noting that neurotoxicity of amyloid beta peptide depends on its conformation and aggregation state as well as on its final concentration and duration of the treatment [1, 31, 32]. Our results indicated that amyloid beta peptide exclusively in aggregated form had a stimulatory effect on PARP activity. Numerous data showed a strong Aβ neurotoxicity both in *in vitro* and *in vivo* experiments [1, 5, 31, 33–36]. However, other authors reported that Aβ produced but a small neurotoxic effect [4, 28, 30, 37, 38]. Our results suggest that glutamate receptor(s) may be involved in Aβ evoked PARP activity. However, when amyloid peptide acted together with NMDA the stimulatory effect of NMDA on PARP activity was not increased in adult hippocampus. Gray & Patel [39] showed that amyloid beta did not alter the potency of glutamate excitotoxicity in cultured neurones exposed to Aβ peptide 25–35 together with glutamate. We showed that the effect of Aβ peptide on PARP activity in adult hippocampus was decreased by the inhibitor of NO synthase. This implies that Aβ, through NO$^\bullet$ and probably other free radicals, is involved in activation of PARP in adult hippocampus.

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Amyloid beta protein affects poly(ADP-ribose) polymerase activity in PC-12 cells in culture

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INTRODUCTION AND METHODS. Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is responsible for a post-translational modification of proteins, including PARP itself called poly(ADP-ribosyl)ation. It is a nuclear, DNA dependent enzyme that consumes cellular NAD^+ to produce chains of ADP-ribose. Its biological role(s) is not yet fully understood, but numerous studies imply its role in DNA repair and other cellular responses to DNA damage (1). The mechanisms by which amyloid beta protein (Aβ) mediates cell death, or the question whether Aβ is cytotoxic *in vivo* in Alzheimer's disease are issues which are not yet solved. The aim of this study was to investigate the effects of amyloid beta peptides on PARP activity and cell death in pheochromocytoma cells (PC-12) in culture. The PC-12 cells nuclei were visualized using Hoechst 33258 staining and their viability was evaluated under a fluorescent microscope. DNA samples were electrophoresed through 1,1% agarose gel and DNA bands were visualized by staining with ethidium bromide. PARP assay was carried out as described previously by Ueda et al. (2) with authors modifications. Aβ fragment 25-35 after aggregation (1h incubation at 37^oC) was added to the cell culture at a final concentration from 1 to 100 μM. The cells were exposed for 2, 6, 48 h to the action of amyloid. As a control reverse sequence 35-25 of Aβ was used.

RESULTS AND DISCUSSION. A significant effect of amyloid beta action was observed after 48 h (Fig.1). After that time even 1 μM Aβ (25-35) caused activation of PARP but significant stimulation of PARP activity was observed at 10 μM Aβ as compared with control samples (Fig.1). Interestingly at 20, 30, and 100 μM of Aβ (25-35), PARP activity was inhibited. The above effects of Aβ (25-35) were pH dependent (Fig.1). PARP activity in untreated PC-12 cells was also pH dependent, meaning it was about 50 % higher at pH 9.0 than at pH 8.0. We also noted that under the same conditions, endogenous activity(ies) of phosphodiesterase(s), an enzyme which takes part in degradation of ADP-ribose chains, was more than two times lower at pH 9.0 than at pH 8.0. In addition, the Hoechst staining revealed that only a few (<5%) apoptotic cells were observed after treatment with Aβ (25-35) (48 h, 10 μM) in contrast to staurosporine treatment (48 h, 0.1μM), which promoted many cells to undergo apoptotic changes. Furthermore, an analysis of DNA integrity using agarose gel electrophoresis revealed, that Aβ (25-35) treatment of PC-12 cells did not result in DNA laddering which is one of the apoptosis markers. Our study indicates a possible link between PARP and amyloid beta action *in vivo*, but the underlying mechanism(s) and its biological role remains unclear and needs further investigation.
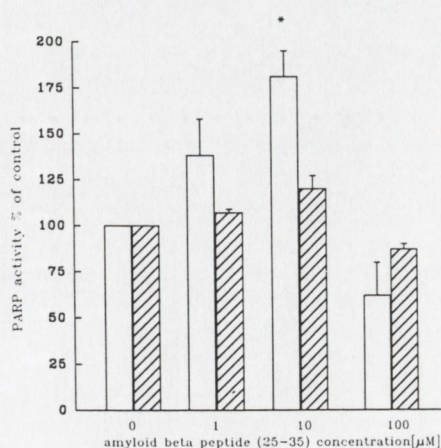

Fig. 1. Effect of Aβ (25-35) on PARP activity in PC-12 cells. PARP activity was determined at pH 8.0 (open bars) and at pH 9.0 (hatched bars). The control activity of enzyme was at pH 8.0 *ca.* 3.57 nmol/mg protein/min and at pH 9.0 *ca.* 5.20 nmol/mg protein/min. Incubation time with Aβ(25-35) was 48 h. The data are mean ± SD from 3-4 experiments carried out in triplicate. Statistical significance was evaluated by Student *t*-test, *$P<0.05$.

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Poly(ADP-Ribose) Polymerase During Reperfusion After Transient Forebrain Ischemia

Its Role in Brain Edema and Cell Death

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Abstract

The activation of poly(ADP-ribose) polymerase (PARP) in the reperfused brain after ischemia has been assumed but never has been directly presented. Our studies indicate a different dynamic of PARP activity alteration in hippocampus during reperfusion after 3 and 10 min of transient forebrain ischemia in gerbils. The phasic stimulation of PARP activity was observed during reperfusion 15 min, 120 min, and 4 d after 3 min of ischemia with subsequent lowering of its activity close to control value on the seventh day of reperfusion. After 10 min of ischemic insult, PARP activity significantly increased from the third to the seventh day of reperfusion. The protein level of PARP was not significantly changed during reperfusion after 3 and 10 min of ischemia, with one exception: On the third day after 10 min of ischemia, PARP protein level was 28% lower compared to control; however, no enhancement of 85-kDa protein immunoreactivity was observed. These data indicate the lack of PARP cleavage in hippocampus of gerbils subjected to ischemia-reperfusion injury. The inhibitor of PARP, 3-aminobenzamide (3-AB) in a dose of 30 mg/kg b.w. (body weight) injected intravenously directly after 3 min of ischemia protects >60% of neuronal cells against death in the CA1 layer of hippocampus but has no effect after 10 min of ischemic episode. 3-AB decreased forebrain edema significantly after 3 and 10 min of ischemia. Our data indicate that PARP inhibitor(s) might offer a potent therapeutic strategy for short global ischemia. The combination of PARP inhibitor with potent antioxidant might enhance its ameliorating effect.

Index Entries: Brain ischemia; poly(ADP-ribose) polymerase; 3-aminobenzamide; neuroprotection.

Introduction

Poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30), a nuclear enzyme, is involved in a variety of physiological and pathological events such as DNA replication, DNA repair, gene expression, cellular differentiation, chromatin decondensation, malignant transformation, and apoptosis (Gaal et al., 1987; Herzeg and Wang, 2001; Satoh and Lindahl, 1992; Ziegler and Oei, 2001; Smith, 2001). In response to DNA damage, PARP is activated and transfers long branched polymers of ADP-ribose to chromatin-associated proteins, utilizing nicotinamide adenosine dinucleotide (NAD) as its substrate. During ischemic stroke, there is a significant increase of extracellular glutamate that causes neuronal damage through events determined as an excitotoxicity. Nitric oxide (NO) produced by neuronal NO synthase (nNOS) plays a preferential role in N-methyl-D-aspartate (NMDA) receptor-mediated neurotoxicity observed in primary brain cultures (Dawson et al., 1991) and in brain ischemia (Chal-

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tological examination, brain ischemia was induced for 3 and 10 min and animals were treated with 3-AB as described below and allowed to survive for 7 d after ischemia.

Treatment with 3-AB

3-AB was dissolved in 0.9% sodium chloride and administrated intravenously (iv) in a dose of 30 mg/kg b.w. (body weight), 10 or 30 min before an insult of 3 or 10 min of ischemia or directly after ischemia. Moreover, some groups of animals were treated with a double dose of 3-AB, 10 or 30 min before ischemia and directly after ischemia (total dose 60 mg/kg b.w.). In the case of 10 min of ischemia, 3-AB was additionally administrated for 60 min and 24 h after ischemia in a dose of 30 mg/kg b.w. Body temperature was kept at 37°C using the same controlled heating system as described previously.

Treatment with MK-801

MK-801 was dissolved in 0.9% sodium chloride and injected intraperitoneally (ip) in a dose of 0.8 mg/kg b.w. in a volume of 0.25 mL, 30 min before ischemia. Body temperature was kept at 37°C using the same controlled heating system as described.

Measurement of Brain Water

Brain edema was determined by comparison of wet and dry weight. Gerbils (four to five animals in each group) were killed 7 d after ischemia. After decapitation, brains were quickly removed and the wet weight of the forebrain was determined on a chemical balance within 90 s after decapitation. After drying the brains in an oven at 100°C for 7 d, the dry weight was obtained. The water content of each hemisphere was calculated as follows:

$$\text{water content (\%)} = \text{wet weight} - \text{dry weight}/ \text{wet weight} \times 100$$

Histological Assessment

For the histological examination, several groups of animals (six to seven animals in each group) were used. 3-β in a dose of 30 mg/kg b.w. was injected iv 30 or 10 min before or directly after 3 or 10 min of ischemia. In the other experiments 3-AB was injected iv 10 or 30 min before ischemia and additionally directly after ischemia, in each case 30 mg/kg b.w. (total dose of 3-AB 60 mg/kg b.w.). Seven days after brain ischemia, animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4. The brains were rapidly removed and postfixed in the same perfusion solution for 7 d before paraffin embedding. Paraffin sections (10 μm thick) at the level of dorsal hippocampus were cut on a microtome and stained with cresyl violet. Surviving neurons in the hippocampal CA1 region were counted under a microscope (magnification, ×200–400).

Determination of PARP Activity

PARP activity was determined using [adenine-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 μL contained 200 μM β-NAD and 4×10^5 dpm [adenine-^{14}C]NAD, 100 mM Tris/HCl buffer (pH 8.0), 10 mM MgCl$_2$, 5 mM DTT, 50 μM P-APMSF, and 100 μg of protein. The mixture was incubated for 1 min at 37°C, and the reaction was stopped by adding 0.8 mL of ice-cold 25% trichloroacetic acid (TCA). Precipitates were collected on Whatman GF/B filters, washed three times with 5% TCA, and left overnight for drying. The radioactivity was determined using a Wallach 1409 scintillator counter (LKB).

Western Blot Analysis

For Western blot analysis, brain homogenates from hippocampus were used. Samples were denaturated for 5 min in sample buffer containing β-mercaptoethanol, then 30 μg of protein per lane was loaded onto an SDS-polyacrylamide gel (using discontinuous gel consisting of stacking [4%] and separating [7.5%] gels) and electrophoresed under a constant 170 V for 45–60 min in an ice-cooled apparatus. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane at 100 V for 1.5 h at 4°C in Dunn buffer (pH 9.9). The membrane was left overnight in blocking solution (5% milk [fat free] in PBS-T) with shaking. Next the membrane was incubated at room temperature with primary antibody solution (monoclonal anti-PARP-1 antibody C-2-10 in 0.25% milk) for 2 h. The membrane was washed three times for 15 min in PBS-T and incubated with secondary antibody HRP-linked anti-mouse immunoglobulin in 0.25% milk for 60 min. Subsequently, membranes were washed three times for 15 min in PBS-T and once for 15 min in PBS. After soaking in ECL, the membrane was exposed to ECL film (Kodak). Immunoreactivity was analyzed using a digital camera and Total Lab 1.1. software (Phoretix).
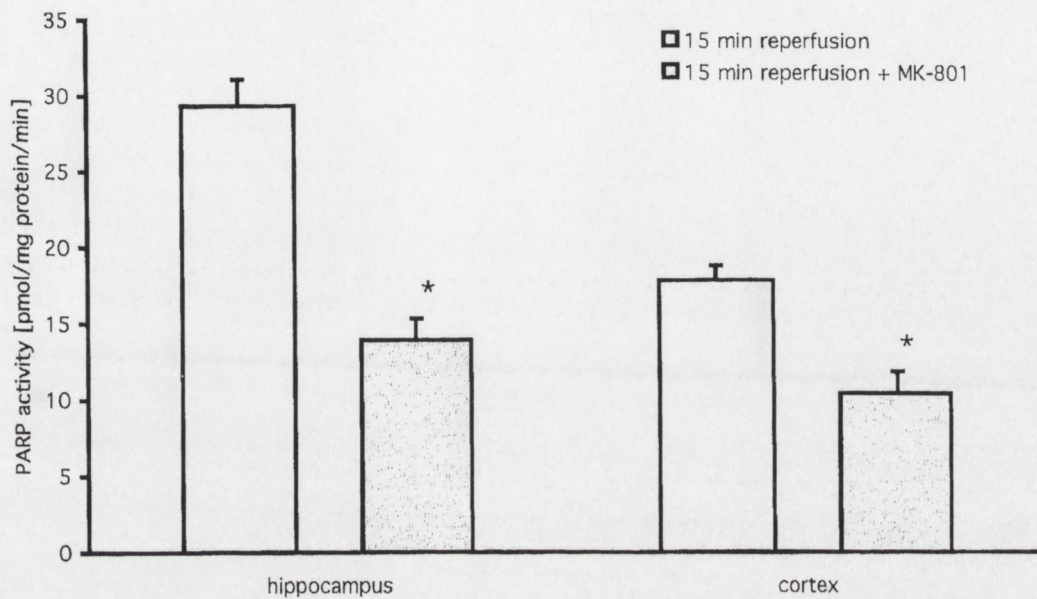
Fig. 2. Effect of MK-801 on PARP activity after 3 min of ischemia and 15 min of reperfusion in gerbil hippocampus. Values are means ±S.E.M. from four to five animals. The results are statistically significant by Student's *t*-test. p (*) < 0.05.

NMDA receptor antagonist MK-801, administrated ip in a dose of 0.8 mg/kg b.w. 30 min before ischemia (3 min), significantly decreased PARP activity, which was enhanced by ischemia-reperfusion in hippocampus. Basal PARP activity in brain cortex was also decreased. (Fig. 2).

Western Blot Analysis of PARP Protein During Reperfusion After 3 and 10 Min of Ischemia

Using MAb C-2-10 and Western blot analysis, we did not find a significant alteration of PARP protein levels during reperfusion after 3 min of ischemia. However, on the second and third day of reperfusion after 10 min of ischemia, the level of PARP protein decreased by 15–28%, and on the seventh day of reperfusion it returned to control value (Fig. 3). There was no enhancement of immunoreactivity in the 85-kDa protein, indicating that the cleavage product of PARP degradation by caspases in the 85-kDa fragment is not present.

Effect of 3-AB on Neuronal Morphology in Histological Examination

The histological examination indicated that 3-AB injected iv 10 min before or directly after 3 min of ischemia (as described in Material and Methods) protected a significant population of neurons against ischemia-reperfusion-induced cell death (Fig. 4). The

results were statistically significant only when 3-AB was injected directly after ischemia (Fig. 4) The number of neurons of 1 mm length in the CA1 layer consists of 310 ± 10 neurons, calculated in 14 fields from 7 control brains; this value was taken as 100%. After 7 days of reperfusion, short forebrain ischemia (3 min) caused cell death in 75–76% neurons and 10 min of ischemia induced cells death in 95–100% neurons (Figs. 4 and 5).

3-AB administered iv in a dose of 30 mg/kg.b.w. before and directly after ischemia had a beneficial effect on CA_1 pyramidal cell survival only after 3 min of ischemia (Figs. 4A and 5). The protective effect of 3-AB strongly depended on the way the drug was administered and the dose and sequence of the drug application. The most pronounced effect was observed after iv application of 3-AB in a dose of 30 mg/kg b.w. directly after ischemia. 3-AB administrated directly after ischemia or 10 min before ischemia resulted in survival of 62% or 58% of neurons, respectively, as compared to control (Fig. 5). However, when 3-AB was injected in a dose of 30 mg/kg b.w. before and after ischemia (altogether 60 mg/kg b.w.), there was no protective effect of 3-AB (Fig. 5). After 10 min of ischemia, the protective effect of 3-AB was not observed (Fig. 4B). Moreover, when 3-AB was administrated 60 min and 24 h after 10 min of ischemia, it had no protective effect (data not shown).
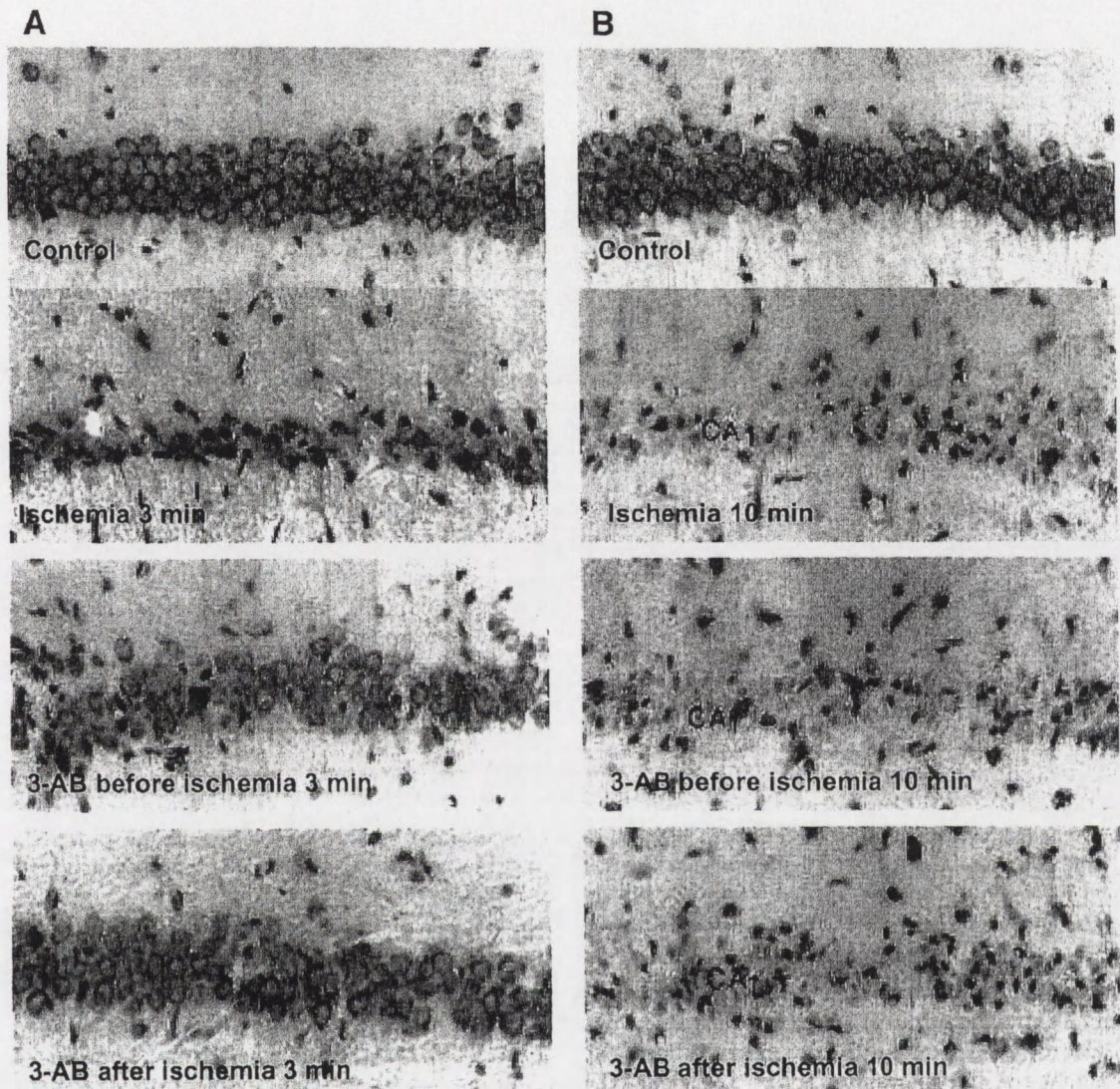
Fig. 4. Histological assessment effect of 3-AB on CA1 pyramidal cell survival after 3 min of transient forebrain ischemia (A) and after 10 min transient forebrain ischemia (B). 3-AB was injected iv in a dose of 30 mg/kg b.w. 10 min before ischemia or directly after ischemia.

ously that excessive stimulation of PARP in brain focal ischemia is deleterious because it causes energy depletion and cell death (Plaschke et al., 2000; Zhang et al., 1994). Recent studies with PARP knockout mice confirm the deleterious role of PARP in brain ischemia (Eliasson et al., 1997; Endres et al., 1997, 1998). These studies clearly show the marked reduction of the infarct volume in PARP knockout mice after transient focal cerebral ischemia. However, presently, there are many controversial results on the role of PARP in global brain ischemia. Results

published by Nagayama et al. (2000) suggest that activation of PARP in rat hippocampus may contribute to cellular recovery following sublethal transient global ischemia. The studies were carried out in rats, in which the four-vessel occlusion technique and hypotension induced brain ischemia. In the study of Nagayama et al. (2000), the cellular NAD level was not significantly altered at any time-point. Furthermore, systemic administration of 3-AB in a dose of 30 mg/kg b.w. prevented the increase in PARP activity at 1 and 24 h of reperfusion and sig-
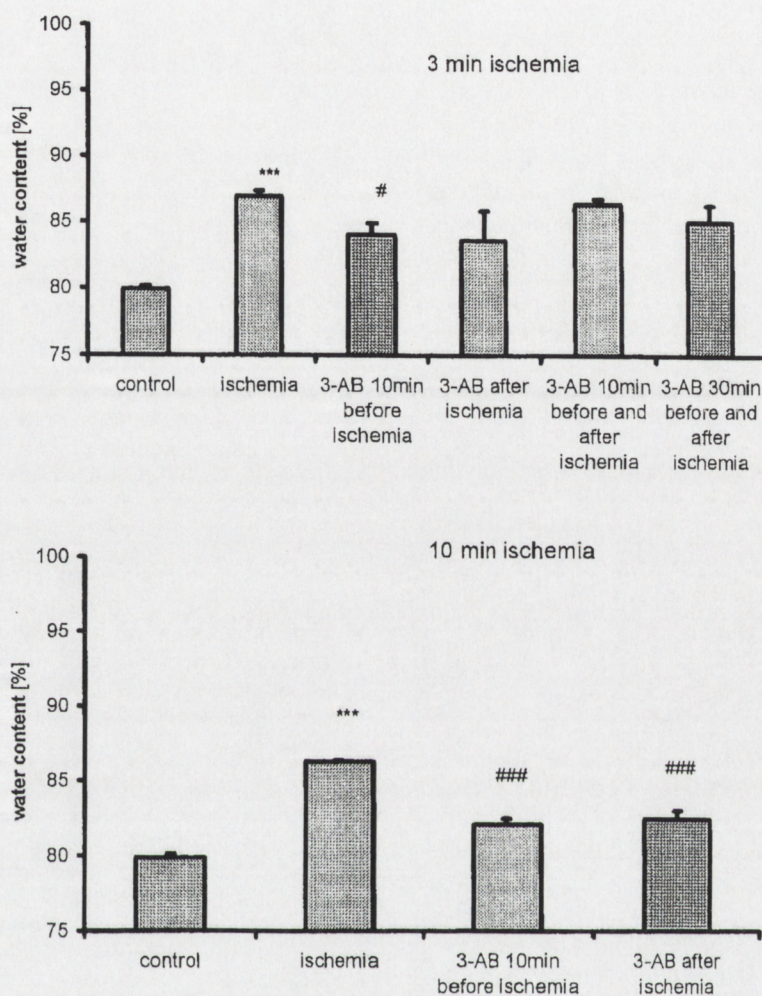
Fig. 6. Effect of 3-AB on water content in gerbil forebrain on the seventh day of reperfusion after 3 and 10 min of ischemia. Values are means ±S.E.M. from four to five animals and are statistically significant ([***] $p < 0.001$) as compared to control ([#] $p < 0.05$) and ([###] $p < 0.001$) compared to ischemia. Statistical significance was evaluated by Student's t-test.

dose of 3-AB (20 mg/kg b.w.) was less neuroprotective. However, their results are in agreement with the studies of Tokime et al. (1998) in which the most effective inhibitor concentration was accorded to 10 mg/kg. b.w. Benzamide and its derivatives have been reported to have the effect on cell viability, glucose metabolism, and *de novo* DNA synthesis. These compounds inhibited activity of nicotinamide-*N*-methyltransferase at high concentrations (Johnson, 1981; Milam and Cleaver, 1984). These side effects might counteract the neuroprotective effect of PARP inhibition. An U-shaped dose response curve for PARP inhibitors is suggested because the majority

of inhibitors were designed to interfere with NAD binding to PARP, and thus PARP inhibitors would inhibit a variety of NAD-dependent enzymes including other isoforms of PARP (Plaschke et al., 2000; Prasad et al., 1999). Specific PARP inhibition and PARP knockout mice might create conditions for better understanding the role of PARP in cell death evoked by brain ischemia-reperfusion injury and hypoxia-reoxygenation, including in vitro oxygen glucose deprivation model. Both necrotic and apoptotic neuronal death have been described following cerebral ischemia (Lipton, 1999; Martin et al., 1998; Nicotera et al., 1999). In this study we found that the

Martin L. J., Al-Abdulla N. A., Brambrink A. M., Kirsch J. R., Sieber F. E., and Portera-Cailliau C. (1998) Neurodegeneration in excitotoxicity, global cerebral ischemia, and target deprivation: a perspective on the contributions of apoptosis and necrosis. *Brain Res. Bull.* **46**, 281–309.

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R.P. STROSZNAJDER[1], H. JESKO[2], A. ADAMCZYK[2]

POLY(ADP-RIBOSE) POLYMERASE-1 IS A NOVEL NUCLEAR TARGET FOR CHOLINERGIC RECEPTOR SIGNALING IN THE HIPPOCAMPUS

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Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear enzyme involved in DNA repair and transcription regulation. The aim of this study was to investigate the role of PARP-1 in muscarinic cholinergic receptor signaling. Our data indicate that activation of muscarinic cholinergic receptors by carbachol (1mM) in the presence of GTPγS evoked a significant enhancement of PARP activity in the adult rat hippocampus. Moreover, TMB-8 (10µM), an antagonist of inositol 1, 4, 5 trisphosphate (IP$_3$) receptor prevented the activation of PARP-1, which indicates that IP$_3$ /Ca^{2+} signaling is involved in this pathway. The diacylglycerol (DAG)-regulated protein kinase C (PKC) inhibitor (GF109203X) (1µM) only slightly enhanced PARP activity in hippocampal nuclear fractions, which suggests that DAG/ PKC is not involved in PARP activation.

Key words: *DAG, IP$_3$, muscarinic receptor signaling, PARP-1, PKC*

INTRODUCTION

Poly(ADP-ribose) polymerase-1 (PARP-1 EC 2.4.2.30) is a highly conserved enzyme localized mainly to the cell nucleus and responsible for over 90% of poly(ADP-ribosyl)ation in the brain (1). PARP-1 activated by single and double DNA strand breaks is the earliest and most sensitive sensor of DNA damage. The overactivation of PARP-1 by massive DNA damage may lead to depletion of intracellular βNAD$^+$ and ATP and to cell death (2-6). Recent studies demonstrate that neuronal PARP-1 can also be stimulated in physiologic conditions, by a fast signal evoked in the cell by membrane depolarization (7, 8). It has been documented that DNA damage is not involved in this pathway. Upon activation,

pH 7.4, without CaCl$_2$, for 30 min at 37°C, followed by another 30-min incubation under carbogen (95% O$_2$ and 5% CO$_2$) in the presence of CaCL$_2$ (2mM), carbachol (1mM), a nonhydrolysable analog of acetylocholine, and with GTPγS (100 μM), a non-hydrolyzable analog of guanosine 5'trisphosphate, an activator of G protein. The IP$_3$ receptor antagonist 8-(Diethylamino)octyl 3,4,5-trimethoxybenzoate hydrochloride (TMB-8) (10 μM) was added to selected samples 5 min before incubation. After incubation, the slices were homogenized and nuclear fractions were isolated, as described by Strosznajder et al (13). In the experiments with a protein kinase C inhibitor, nuclear fractions were incubated for 15 min with or without GF 109203X at a final concentration of 1 μM and the PARP-1 activity was determined using a radiochemical methods.

Determination of PARP activity

PARP activity was determined using [adenine-14C]NAD as substrate. The incubation mixture in a final volume of 100 ml contained 200 μM ßNAD and 2 x 10^5 dpm [adenine-^{14}C]NAD, 100 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl$_2$, 5 mM DTT, 50 μM p-APMSF, and 100 μg protein. The mixture was incubated for 1 min at 37 °C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitates were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. Afterwards, after adding Bray liquid scintillation cocktail, radioactivity was measured in a 1409 Wallac scintillation counter (Wallac Oy, Turku, Finland).

Differences in PARP activity were compared with one-way ANOVA followed by the Newman-Keuls *post hoc* test. A value of P<0.05 was deemed to indicate significant differences.

RESULTS

Stimulation of cholinergic receptors in the adult hippocampal slices by carbachol and GTPγS enhanced the activity of PARP-1 by about 100% comparing with the basal, unstimulated condition (*Fig. 1*). The enhancement was eliminated
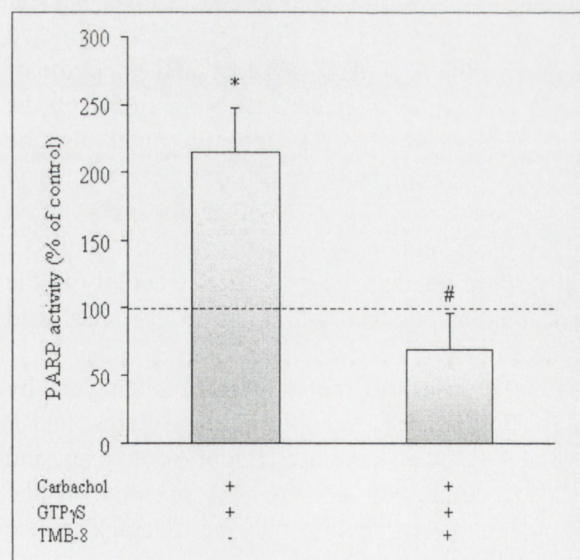

Fig. 1. Effects on PARP-1 activity of cholinergic receptor stimulation in the hippocampal tissue. Stimulation of PARP by carbachol + GTPγS was eliminated by the addition of the IP$_3$ receptor antagonist TMB-8. Data show percentage changes from control, taken as 100% (marked by the horizontal dotted line). *Different from control at P<0.01; #different from carbachol + GTPγS at P<0.05.

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NON-Aβ COMPONENT OF ALZHEIMER'S DISEASE AMYLOID AND AMYLOID BETA PEPTIDES EVOKED POLY(ADP-RIBOSE) POLYMERASE-DEPENDENT RELEASE OF APOPTOSIS-INDUCING FACTOR FROM RAT BRAIN MITOCHONDRIA.

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Amyloid beta peptide (Aβ) and non-Aβ component of Alzheimer's disease amyloid (NAC) are involved in pathomechanism of Alzheimer's Disease (AD) and are deposited in the AD brain in the form of senile plaques. However, the mechanism of their neurotoxicity is not fully understood. In this study the sequence of events involved in NAC and Aβ peptides evoked toxicity was investigated in brain slices, synaptosomes and in subcellular fractions. Radio-, immunochemical, spectrophotometrical methods and DNA electrophoresis were used in this study. Our data indicated that Aβ 1-40 (25 μM) and NAC (10 μM) peptides induced liberation of free radicals and massive DNA damage that lead to activation of DNA bound enzyme poly(ADP-ribose) polymerase-1 (PARP-1). In consequence of these processes apoptosis-inducing factor (AIF) was released from mitochondria and was translocated to nucleus. The inhibitor of PARP, 3-aminobenzamide significantly decreased AIF release from mitochondria and its translocation. Both peptides under the investigated conditions had no effect on caspase-3 activity. Our data indicated that Aβ and NAC peptides stimulate AIF-dependent apoptotic pathway that seems to be caspase independent process. The inhibition of PARP-1 may protect the brain against Aβ and NAC toxicity.

Key words: *amyloid beta peptide, NAC, reactive oxygen species, DNA degradation, apoptosis-inducing factor*

Preparation of synaptosomes.

Synaptosomes were prepared as previously described (12). Cerebral hemispheres were cut into small fragments and homogenized in a solution containing 0.32 M sucrose, 2mM EDTA, 2mM EGTA, 20mM HEPES and a cocktail of protease inhibitors. The homogenate was centrifuged for 3 min at 900 x g at 4°C, and the supernatant was then centrifuged for 10 min at 12 400 x g at 4°C. The pellet was resuspended in 1.5 ml of homogenisation buffer and centrifuged through a discontinuous sucrose gradient (7ml 1.18M sucrose, pH 8.5; 7ml 1M sucrose, pH 8.0; 7ml 0.85 M sucrose pH 8.0) at 87 275 x g for 2 h. Synaptosomes in the 1M sucrose/1.18M sucrose interface were removed, resuspended in 4 vol of Locke's solution (NaCl, 154mM; KCl 5.6mM; $CaCl_2$ 2.3mM; $MgCl_2$ 1.0mM; $NaHCO_3$ 3.6mM glucose 5mM; Hepes, 5mM; pH 7,2) and centrifuged for 10 min at 17 000 x g at 4°C.

Preparation of nuclear and mitochondria fractions

Brain slices were homogenized in 20 mM HEPES buffer, pH 7.5 containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.32 M sucrose and proteases inhibitors complete and centrifuged for 5 min at 500 x g at 4°C. The resulting supernatant (S_1) was centrifuged for 20 min at 10 000 x g at 4°C to yield cytosol (S_2) and mitochondria (P_2). Pelleted nuclei (P_1) were suspended in lysis buffer (10mM Tris-HCl, pH 7.4, 100 mM NaCl, 0,1 mM EDTA, and protease inhibitors complete) and incubated in ice for 15 min. Then centrifuged for 20 min at 15 000 x g at 4°C to yield nuclear extract.

Preparation of Aβ and NAC peptides

Aβ 1-40 was prepared by dissolving it in sterile water (vehicle) at 250 µM concentration and used either immediately in non-aggregated form or incubated at room temperature to obtain the aggregated peptide form as described previously (11).

NAC peptide was stored lyophilized, and 100µM stock solution was prepared in sterile water and then used at final 10µM concentration in the soluble form or incubated for 3 days at 37°C to obtain the aggregated form.

Measurement of ROS production

Intrasynaptosomal generation of ROS was measured using fluorogenic probe, 2'7'-dichlorofluorescin diacetate (also known as 2'7'-didichlorodihydrofluorescein diacetate; DCFH-DA). DCFH-DA is intracellularly deacetylated to 2'7'-dichlorofluorescin (DCFH) and then oxidized by hydrogen peroxide to a fluorescent compound, 2'7'-dichlorofluorescein (DCF). Freshly isolated synaptosomes suspended in Locke's buffer were mixed with DCFH-DA at 15 µM concentration and incubated for 120 min at 37°C under carbogen in the presence of NAC and Aβ peptides, both in soluble and aggregated form at 10 µM and 25 µM concentration, respectively and with H_2O_2 at 100 µM in the dark. The concentration of DCF was measured by a fluorescence spectrophotometer with excitation at 488 nm and emission at 530 nm.

Determination of PARP activity

Brain slices were preincubated for 30 min at 37°C under carbogen. Then treated with NAC and Aβ peptides at 10 µM and 25 µM concentration, respectively for 30 min at 37°C under carbogen. After incubation the brain slices were homogenized and PARP activity was determined using adenine[^{14}C]NAD as substrate. The total reaction mixture in final volume of 100 µl contained 200

St. Louis, MO, U.S.A.). Then blots were washed as described above and bound antibodies were visualized by enhanced chemiluminescence (Amersham, U.K.). Quantification of immunoblots was performed with the NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

RESULTS

The effect of Aβ and NAC peptides on generation of ROS was measured in nerve endings fraction (synaptosomes) using the fluorogenic probe, DCFH-DA. Production of DCF, equivalent of ROS generation, was determined after 2 h treatment of synaptosomes with 10 µM NAC peptides in soluble and aggregated form. Moreover, synaptosomes were incubated in the presence of soluble and aggregated Aβ 1-40 at 25 µM. Hydrogen peroxide (H_2O_2) at 100 µM concentration was used as a control. Aggregated and soluble NAC peptides significantly activated ROS generation by 42% and 33%, respectively (*Fig 1*). However, Aβ peptide exclusively in aggregated form activated free radicals formation by 33% comparing to control.

Aggregated NAC and Aβ peptides at 10 µM and 25 µM concentration, respectively but not soluble form induced significant DNA fragmentation (*Fig 2A,*
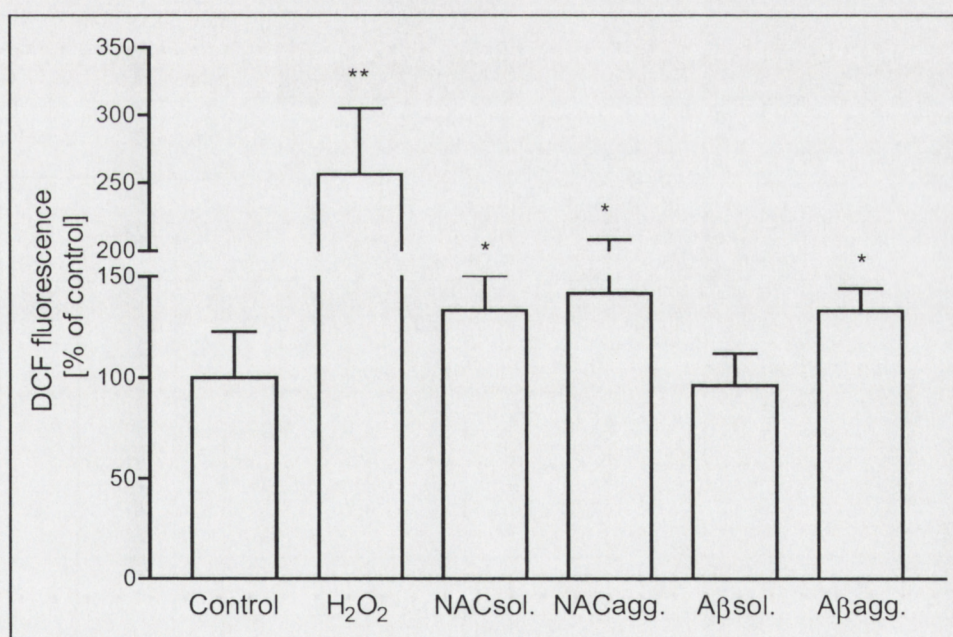
Fig. 1. Intrasynaptosomal generation of ROS after treatment with NAC and Aβ peptides.
ROS generation was measured in synaptosomes after 2-h incubation in Locke's buffer equilibrated with 5% CO_2 in 95% O_2 at 37°C with 10 µM NAC and 25 µM Aβ 1-40 (soluble and aggregated) or without these compounds (control) using fluorescence probe DCFH. Data represent the mean value ± SEM of four separate experiments. *p<0.05 by paired *t*-test between control and the experimental condition.
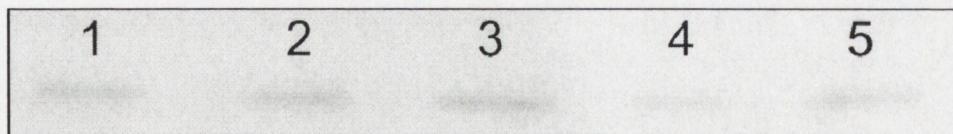
Fig. 4. NAC and Aβ peptides-induced AIF release from mitochondria and its translocation to nucleus. Effect of 3-AB.
Western blot analysis of AIF expression in nuclear fraction isolated from control brain slices (*lane 1*) and incubated in the presence of aggregated Aβ 1-40 (*lane 2*), aggregated NAC (*lane 3*), aggregated Aβ 1-40 + 3-AB (*lane 4*) and aggregated NAC + 3-AB (*lane 5*). NAC was used at 10 μM and Aβ at 25 μM concentration, respectively and 5 mM 3-AB.

2B). Enhancement of PARP-1 activity is the earliest and most sensitive indicator of DNA damage. Aggregated NAC and Aβ peptide significantly stimulated PARP activity by about 20% and 87%, respectively (*Fig 3*). Both peptides at soluble form had no effect on this enzyme activity. Our results indicated that NAC and Aβ peptide exclusively in aggregated form evoked AIF release from mitochondria and its translocation to nucleus. Inhibitor of PARP, 3-aminobenzamide (3-AB) at 5mM concentration decreased AIF release and prevented its translocation (*Fig 4*). Both peptides under the same conditions have not effect on caspase-3 activity (data not shown).

DISCUSSION

Amyloidogenic peptides, i.e Aβ and NAC, which form a β-pleated sheet structure are involved in neurodegeneration. The ability of Aβ peptide to induce oxidative stress and apoptosis-related events was described previously (12, 13). The neurotoxicity of NAC was originally reported by Liu and Schubert (14) in rat brain tumor B12 cells, and then in human neuroblastoma SH-SY5Y cells (15). However, the mechanism of this neurotoxicity is not fully understood. It was put forward that NAC and Aβ activate ROS production and play a major role in neurodegenerative disorders (7, 13). Our data demonstrated that Aβ and NAC peptides activated ROS generation and induced massive significant DNA fragmentation after 2 h treatment of rat brain slices. Enhanced generation of free radicals by these peptides might be responsible for DNA damage. PARP-1 is the earliest indicator of DNA damage (16). Stimulation of PARP-1 by aggregated Aβ peptides was observed in our previous studies in brain and PC12 cells (17, 18). In the present study, we showed that PARP-1 was also significantly activated by aggregated NAC peptides. Recent data point to this enzyme as an important activator of apoptotic cell death (19, 20, 21). Increased activity of PARP-1 leads to depletion of βNAD$^+$ and ATP and to lowering of mitochondrial membrane potential. These may trigger the release of AIF from mitochondria before cytochrome c release and caspase activation does occur. The translocation of AIF

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Effect of carvedilol on neuronal survival and poly(ADP-ribose) polymerase activity in hippocampus after transient forebrain ischemia

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Abstract. Carvedilol a β-adrenoreceptor antagonist with potent antioxidant properties raises high expectations in therapy of ischemia. In this study the effect of carvedilol on neuronal survival after transient forebrain ischemia in gerbils was investigated. The role of poly(ADP-ribose) polymerase (PARP-1) in this process was evaluated. Our data indicated that carvedilol administered subcutaneously in a dose of 7 or 70 mg/kg b.w. directly after 5 min of transient forebrain ischemia protects significant population of neurons in hippocampal area CA1, but has no effect after induction of prolonged 10 min ischemia. Carvedilol significantly decreased PARP activity in hippocampus that was markedly increased after both 15 min and 4 days of reperfusion following 5 min of ischemia. Moreover, carvedilol prevented NAD^+ depletion after ischemic-reperfusion insult. These results indicated that carvedilol protects neurons against death and suggested that suppression of PARP activity during reperfusion could be involved in this process.

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Key words: carvedilol, PARP, ischemia, reperfusion, neuroprotection

pH 7.4 together with protease inhibitors Complete. Then homogenates were quickly frozen at -70°C and then used for biochemical determination.

Treatment with carvedilol

The animals were injected subcutaneously in a dose of 7 and 70 mg /kg b.w. directly after ischemia and allowed to survive 15 min, 4 and 7 days after ischemic episode. Carvedilol was dissolved in 100% DMSO then diluted in 0.9% saline to achieve the final concentration of 50 % DMSO in saline. The volume of administered solution did not exceed 200 μl.

Histological assessment

For the histological examination several groups of animals (6–7 animals per group) were used. Carvedilol in a dose of 7 or 70 mg/kg b.w. was injected subcutaneously directly after 5 or 10 min ischemia. Seven days after brain ischemia animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline pH 7.4. The brains were rapidly removed and postfixed in the same perfusion solution for 7 days before paraffin embedding. Paraffin sections at the level of dorsal hippocampus 10 μm thick were cut on a microtome and stained with cresyl violet. Neurons in the hippocampal CA1 region were counted under the 200–400× magnification in the light microscope. In each group the average number of neurons per 1 mm length of section through the area CA1 was counted.

Preparation of brain homogenate and nuclear fraction

Brain homogenate (10%) from cerebral cortex and hippocampus was prepared in 0.32 M sucrose with Tris-HCL pH 7.4, 1 mM EDTA using Dounce glass homogenizer. To obtain crude nuclear fraction, homogenate was centrifuged at 900 g for 3 min and the obtained pellet was used for the PARP assay.

Determination of poly(ADP-ribose) polymerase activity

PARP activity was determined using [adenine-^{14}C]NAD as a substrate. The incubation mixture in a final volume of 100 ml contained 200 μM βNAD and 2 × 10^5 dpm [adenine-^{14}C]NAD; 100 mM Tris–HCl

buffer (pH 8.0); 10 mM $MgCl_2$; 5 mM DTT; 50 μM P-APMSF and 100 μg of protein. The mixture was incubated for 1 min at 37°C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitate were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. The radioactivity was measured using scintillator counter LKB, Wallach 1409.

Determination of NAD$^+$ concentration

NAD$^+$ concentration was determined according to the method of Nisselbaum and Green (1969) modified by Stern (Stern et al. 2002). Animals were quickly decapitated and the heads were put in liquid nitrogen. Then the nitrogen powder were prepared from isolated brain and dispersed in 0.1 M HCL and then incubated at 100°C for 2 min then chilled to 0°C and centrifuged Supernatant was collected and was neutralized and added to the reaction mixture containing 50 mM of Tris-HCL pH 8.0 1mM of phenazinmethosulfate, 0.25 mM of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide, 0.2 mg of alcohol dehydrogenase, 300 mM of ethanol in a total volume of 2 ml. Reaction mixture was incubated at 37°C without ethanol and reaction was started by adding ethanol. The rate of increased absorbance was determined for 2 min at 560 nm. Blanks without NAD were used to correct background rate. NAD$^+$ concentration was calculated from standard curve.

The statistical analysis was performed by one-way ANOVA, using Newman-Keules *post-hoc* test.

RESULTS

Carvedilol protects CA1 hippocampal neurons against death after 5 min forebrain ischemia

Histological examination of hippocampal sections of the brains collected 7 days after ischemia showed that carvedilol injected subcutaneously in a dose of either 7 or 70 mg/kg b.w. directly after 5 min of transient forebrain ischemia protected a significant population of neurons (Fig.1). In the brains of seven control animals, the average number of neurons per 1 mm length of section through the area CA1 was 310 ± 10 and this value was taken as 100%. Without the carvedilol protection about 90–100% neurons of the hippocampal area CA1 died 7 days after 5 min
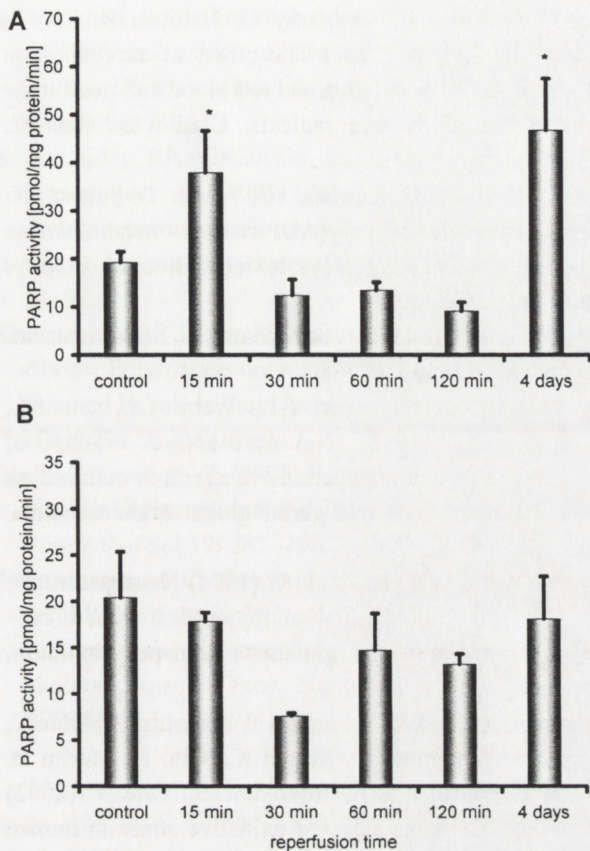
Fig. 3. (A) PARP activity in the hippocampus during reperfusion after 5 min of transient forebrain ischemia. Values are means ± SEM from 3–6 animals in each experimental group. The results are statistically significant at $P<0.05$. (B) PARP activity in cerebral cortex during reperfusion after 5 min of forebrain ischemia in gerbil. Values are means ± SEM from 3–6 animals.
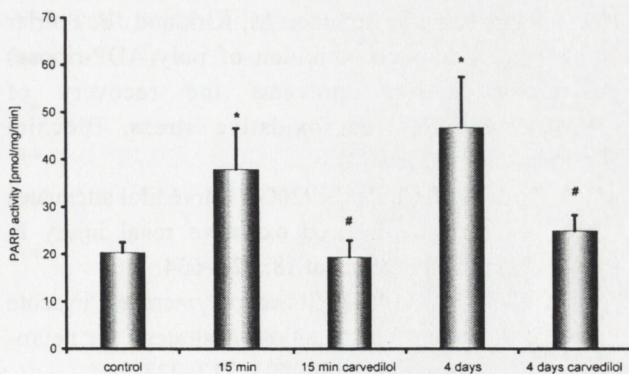

Fig. 4. Effect of carvedilol on PARP activity in the hippocampus of gerbils reperfused for 15 min and 4 days after 5 min ischemia. Values are means ± SEM from 4–5 animals. (*) $P<0.05$ comparing to control, (#) $P<0.05$ comparing to ischemia reperfusion.

ribose) polymerase 3-AB is able to protect more then 50% of neurons in CA1 layer of hippocampus after 3 min but not after 10 min ischemia. Lysko and coauthors (1992) and Habon and coauthors (2001) demonstrated the neuroprotective effect of carvedilol in *in vitro* and *in vivo* experiments. In *in vitro* experiments performed on cultured rat cerebral neurons carvedilol protects the cells in a dose dependent manner against glutamate-mediated excitotoxicity and also against free radicals generation after addition of ferrum ions. In *in vivo* experiments Lysko and coauthors (1992) used the gerbil global brain ischemia model. Carvedilol administered subcutaneously before or/and after ischemic episode had protective effect on CA1 hippocampal neurons. Habon and coauthors (2001) in the experiments performed on ischemic-reperfused hearts presented that carvedilol decreased ROS level and the single-strand DNA breaks and suppressed the self ADP-ribosylation of PARP. Moreover, it was shown that carvedilol in a concentration of 1 µM potently inhibited PARP activity isolated from rat liver. Till now there was no experimental evidences indicated possible interrelation between PARP activity and carvedilol action in postischemic brain. Our data demonstrate that the enhancement of PARP activity during first 15 min of reperfusion might be due to the elevation of oxidative stress and suggest the involvement of PARP in DNA repair mechanism. However, the second rise in PARP activity observed 4 days of reperfusion is probably associated with the massive DNA damage. The PARP over activation may lead to energy depletion and in consequence to cell death. Our results presented in this paper clearly show that carvedilol significantly decreased the PARP activity after short ischemic insults, 15 min and 4 days of reperfusion. Moreover, carvedilol protected ischemic brain against NAD+ depletion.

CONCLUSIONS

We suggest that neuroprotection of CA1 hippocampal neurons after carvedilol treatment my correlate with diminished PARP activity during reperfusion after transient forebrain ischemia. We propose that carvedilol through its action prevents brain cells against PARP-1 over activation and subsequently against depletion of its substrate βNAD+ and ATP exhaustion.

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Inhibition of poly(ADP-ribose) polymerase activity protects hippocampal cells against morphological and ultrastructural alteration evoked by ischemia-reperfusion injury

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Abstract

Poly(ADP-ribose) polymerase 1 (PARP-1 EC 2.4.2.30) is a nuclear enzyme that plays an important role in cell survival and death. PARP is involved in DNA repair machinery, however, massive DNA damage leads to overactivation of PARP-1 and to depletion of its substrate βNAD⁺ which causes cell death. Our previous study indicated that the PARP activity was significantly activated during ischemia-reperfusion injury.

In this study we investigated the effect of PARP inhibitor, 3-aminobenzamide (3-AB) on intracellular organelles alteration. Gerbils were submitted to 3 and 10 min transient global ischemia followed by recirculation and survival for 1 till 7 days. The histological and electron microscopic examination indicated a pronounced protective effect of 3-AB on the swelling of astrocytes and neurons 1 day after 3 and 10 min ischemic insult. It decreased also the swelling of pericytes. 3-AB decreases evoked by ischemia swelling of mitochondria and Golgi apparatus. The significant ameliorating effect of 3-AB was also observed on the 7th day of reperfusion after 3 min ischemia and was also visible on the 1st day after 10 min ischemia. However, 7 days after prolonged 10 min ischemia almost all neurons in the CA1 hippocampal layer died and 3-AB was not able to protect these cells. In spite of that, 3-AB markedly decreased immunostaining of glial fibrillary acidic protein (GFAP), which was enhanced in the stratum: oriens, radiatum and lacunosum-moleculare at the 7th day after 10 min ischemia. These data indicated that inhibition of PARP may have a protective effect on neuronal cells affected by ischemia-reperfusion injury.

Key words: *PARP, ischemia, neuroprotection, 3-aminobenzamide, reperfusion*

Introduction

The family of poly(ADP-ribose) polymerase (PARP) consists of 16 isoenzymes among them the best characterized is PARP-1 (EC 2.4.2.30). This DNA bound enzyme is the most abundant isoform in the brain where it is responsible for more than 90 % of poly (ADP-ribosy) lation processes. PARP plays a key role in nuclear DNA repair and facilitates the repair of simple alkylation damage of the mitochondrial DNA. This enzyme is involved in many cellular processes as gene transcription, chromatin function, genomic stability,

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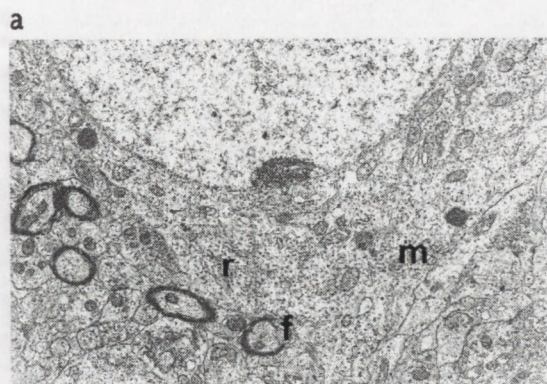e-mail: roberts@cmdik.pan.pl

a

Fig. 2a. Cellular ultrastructure in CA1 hippocampal layer on the 1ˢᵗ day after 3 min of forebrain ischemia. In perikaryon of neuron swelling of the mitochondria (m) and endoplasmatic reticulum (r), disaggregation of ribosomes with delamination of nerve fibers (f) are visible. Magnification x 15000
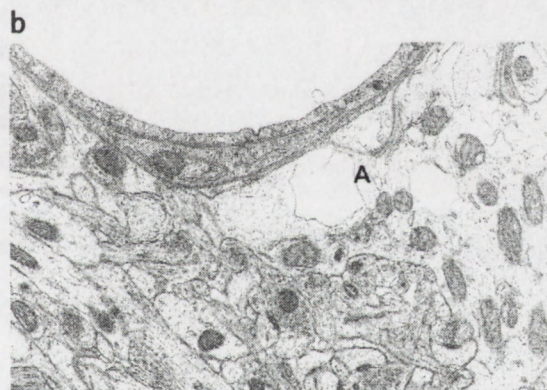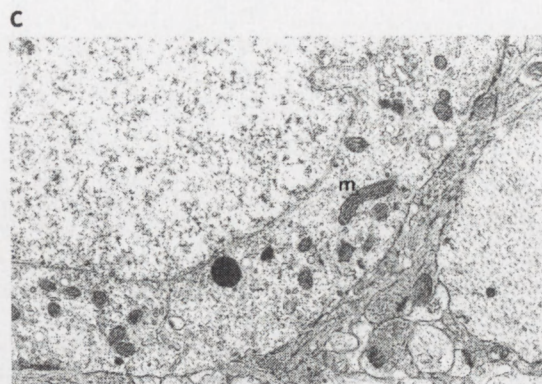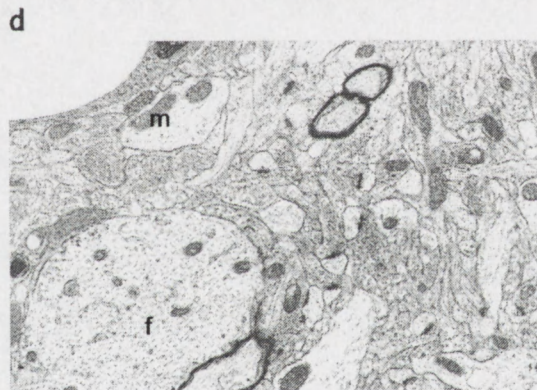
c

Fig. 2c. Cellular ultrastructure in CA1 hippocampal layer on the 1ˢᵗ day after 3 min of forebrain ischemia. Protection with 3-AB. In the perikaryon of the neuron well preserved mitochondria (m), Golgi apparatus and endoplasmatic reticulum are visible. Magnification x 23000

b

Fig. 2b. Cellular ultrastructure in CA1 hippocampal layer on the 1ˢᵗ day after 3 min of forebrain ischemia. In the perivascular zone damage of astrocyte (A) is visible. Magnification x 30 000

d

Fig. 2d. Cellular ultrastructure in CA1 hippocampal layer on the 1ˢᵗ day after 3 min of forebrain ischemia. Protection with 3-AB. In the perivascular space unchanged mitochondria (m), nerve endings and nerve fibers (f) are visible. Magnification x 18000

3-aminobenzamide treatment

3-aminobenzamide was dissolved in 0.9% sodium chloride and was injected intravenously in a dose of 30 mg/kg b. w at final volume of 100 μl, directly after 3 and 10 min of brain ischemia.

Preparation for electron microscopy

For electron microscope examination the brains of anesthetized animals were perfused through the heart with fixative solution 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.4. After removal of the brains, hippocampal samples were taken and fixed in the same solution for 24h at 20°C. Postfixation was completed with 1% osmium tetroxide (OsO_4) and 0.8% potassium ferricyanide $K_4[Fe(CN)_6]$. After dehydration in ethanol and propylene oxide the specimens were embedded in Spurr resin [25]. Ultrathin sections (50 nm) were examined with an electron microscope (JEM 1200 EX, JEOL Japan).
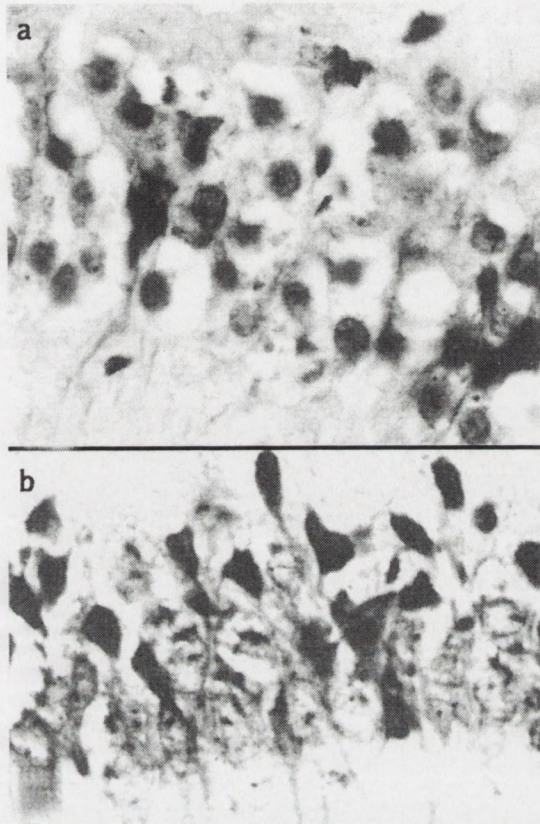
Fig. 5a. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1ˢᵗ day after 10 min of forebrain ischemia. Magnification x 690

Fig. 5b. Morphology of the pyramidal neurons in CA1 hippocampal layer on the 1ˢᵗ day after 10 min of forebrain ischemia. Protection with 3-AB. Magnification x 690

morphology (light microscope) and ultrastructural alteration (electron microscope) in neuronal, glia cells and blood vessels was evaluated. The alteration on the level of cells and intracellular organelles as the nucleus, mitochondria, endoplasmic reticulum and Golgi apparatus was described according to the same criteria.

Morphology and cellular ultrastructure 1 day after 3 min of forebrain ischemia. 3-AB neuroprotection

The histological examinations indicated that one day after 3 min ischemia numerous dark neurons are present in CA1 layer of the hippocampus. The neuropile is significantly altered by edema (Fig. 1a). In the electron microscope (EM) the partial damage of mitochondria, very active Golgi apparatus, swelling of endoplasmatic reticulum, dissagregation of ribosomes were observed (Fig. 2a). Also some nerve endings became swollen with sparse synaptic vesicles, and partial delamination of nerve fibers was visible (Fig. 2a).

Damage of astrocytes with vacuolization and a large drop of lipids was observed (Fig. 2b). In the endothelial cell, activity of pinocytotic vesicles was observed (Fig. 2b). After 3-AB treatment the signs of edema were less expressed, the pyramidal cells were well preserved and only a few dark neurons were observed (Fig. 1b). The protective effect of PARP-1 inhibitor was also observed on the ultrastructure level. EM examination showed that 3-AB protects the neuronal perikaryon. Swelling of mitochondria was not observed (Fig. 2c). The Golgi apparatus and endoplasmatic reticulum were not activated. Also synaptic vesicles were not dispersed (Fig. 2c). Fig. 2d the perivascular space after 3-AB treatment was well preserved. The endothelial cell surface was unchanged. Mitochondria, nerve endings and nerve fibers were in good condition (Fig. 2d).

Morphology and cellular ultrastructure 7 days after 3 min of forebrain ischemia. 3-AB neuroprotection

7 days after 3 min of brain ischemia a significant loss of pyramidal cells in the CA1 layer of the hippocampus was found (Fig. 3a). In non treated rats about 80 % of neurons died (Fig. 3a). EM examination showed pathological changes in the perikaryal zone. Enlargement of the Golgi apparatus, partially damaged mitochondria and vacuolisation of the endoplasmatic reticulum were observed (Fig. 4a). After 3-AB treatment only about 40% of cells died (Fig. 3b). Mitochondria and nerve endings visible in the perivascular area (EM) were well preserved (Fig. 4b).

Morphology and cellular ultastructure 1 day after 10 min of forebrain ischemia. 3-AB neuroprotection

The 10 min of ischemic episode induced more significant alteration of the neurons, glia cells and intracellular organelles comparing to 3 min of
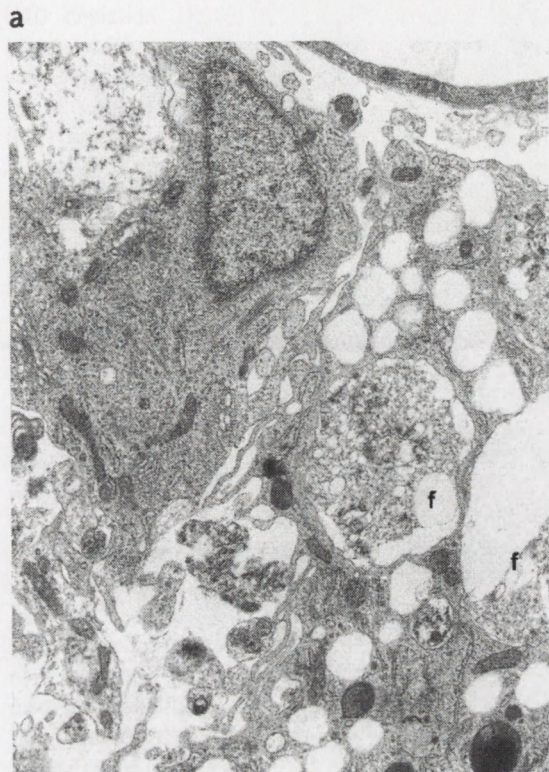
a   **b**

Fig. 8a. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 10 min of forebrain ischemia. Brain parenchyma near the blood vessel is completely destroyed. The macrophage (f) with many phagolysosomes is visible. Magnification x 15000
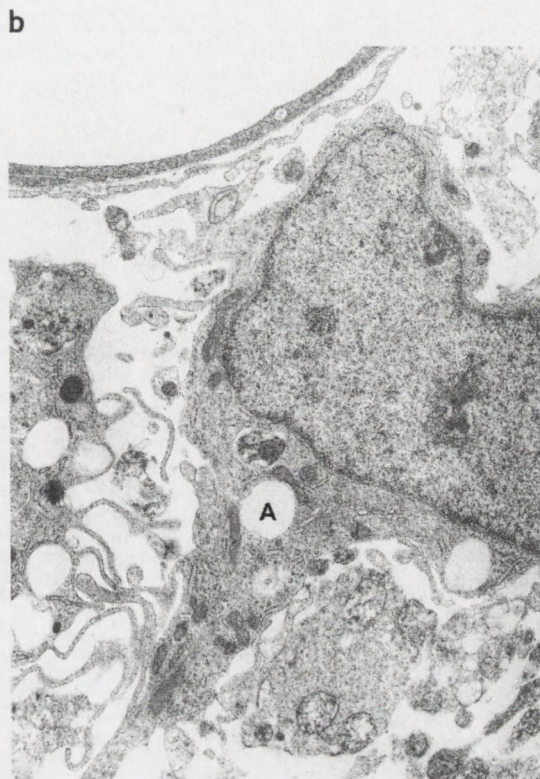
Fig. 8b. Cellular ultrastructure in CA1 hippocampal layer on the 7th day after 10 min of forebrain ischemia. Protection with 3-AB. Brain parenchyma near the blood vessel is completely destroyed. In the center very active astrocyte (A) is visible. Magnification x 15000

CA1 hippocampal cells (Fig 8b). In the CA1 hippocampal layer only fragments of neuronal cells persisted. Nuclear chromatin grouping characteristic of apoptosis was not observed. However, in 3-AB treated group astrogliosis and GFAP expression was weaker (Fig. 9b) comparing to the untreated group where activation of astrocytotic gliosis was observed mainly in the stratum: oriens, radiatum and lacunosum-moleculare of the hippocampus (Fig. 9a). Table 1 presents the most important ultrastructural changes which were found under the electron microscope investigation in all investigated groups. As it was shown, 3-AB had the most protective effect after induction of short 3 min ischemic insult. However, during 10 min ischemia, 1 day of reperfusion its ameliorating effect was also visible.

Discussion

Our present data and previously published preliminary results [19] indicated for the first time the significant protective effect of PARP-1 inhibitor on altered by ischemia cells morphology and ultrastructure of intracellular organelles.

The results demonstrated that 3-AB protected neurons and astrocytes against cytotoxic edema. The evoked by ischemia swelling of mitochondria, endoplasmic reticulum and the Golgi apparatus was significantly less expressed after 3-AB treatment. The necrotic signs of ischemic cell changes were significantly diminished by 3-AB in the CA1 hippocampal layer on the 1st and the 7th day after short 3 min ischemia and also on the 1st day after 10

NAD depletion. However, despite its ameliorating effect which was observed after 5 min ischemia carvedilol was not able to protect the pyramidal neurons in CA1 layer against death after prolonged 10 min ischemia. The data indicated that even such a multipotential drug as carvedilol with potent antioxidant properties is not able to protect neurons against necrotic death after a prolonged ischemic episode. We suggest that during a short ischemic insult the inhibition of PARP is crucial for maintaining NAD and ATP level. 3-AB markedly protects the mitochondria against degeneration as it was shown in the most presented EM graphs. Moreover, our morphological and ultrastructural investigations carried out in the CA1 hippocampal layer after transient forebrain ischemia in gerbils indicated only necrotic processes. There was no evidence for apoptotic cells death. The mitochondria and Golgi apparatus swelling, disaggregation of polyribosomes, and cell and nuclear membrane breaks suggested neuronal necrosis. These observations in our studies are in agreement with Martin at al. [12] and Yamamoto et al. [26]. Also Colbourne et al. [5] presented the EM evidences against apoptosis as the mechanism of neuronal death in global ischemia in gerbils. However, Nitatori et al. [14] suggested that delayed death in the CA1 pyramidal cells after transient ischemia is apoptotic. This point of view is presented also by Moroni group [13] but they never demonstrated morphological and ultrastructural apoptotic alteration in the gerbil model of brain ischemia. The role of PARP-1 in global brain ischemia has not been completely understood as yet and the available data are controversial as it was presented in our last review [20]. Our previous data [18] and these results demonstrate the significant neuroprotective effect of PARP-1 inhibitor on cells degeneration and death exclusively after short forebrain ischemia. These findings may have clinical relevance specially in cardiac arrest and during short ischemic insults. Moreover, PARP-1 is now a major target of biological and medical investigations and a number of data obtained by leading laboratories in this field indicate that PARP-1 inhibitors may be efficient drugs against stroke, inflammation and neurodegenerative diseases. In conclusion, our data clearly show that inhibition of PARP-1 activity had a neuroprotective effect on the neuronal and glial ultrastructure. 3-AB protects the neuronal cells against the necrotic type of death and this neuroprotection is closely correlated with the duration of ischemic-reperfusion episode.

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Effect of aging and oxidative/genotoxic stress on poly(ADP-ribose) polymerase-1 activity in rat brain

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Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30), a DNA-bound enzyme, plays a key role in genome stability, but after overactivation can also be responsible for cell death. The aim of the present study was to investigate PARP-1 activity in the hippocampus, brain cortex, striatum and cerebellum in adult (4 months) and aged (24 months) specific pathogen free Wistar rats and to correlate it with PARP-1 protein level and p53 expression. Moreover, the response of PARP-1 in adult and aged hippocampus to oxidative/genotoxic stress was evaluated. Our data indicated a statistically significant enhancement of PARP-1 activity in aged hippocampus and cerebral cortex comparing to adults without statistically significant changes in PARP-1 protein level. The expression of p53 mRNA was elevated in all aged brain parts with the exception of the cerebral cortex. Our data suggest that enhancement of PARP-1 activity and p53 expression in aged brain may indicate higher DNA damage. Our data also indicate that during excessive oxidative/genotoxic stress there is no response of PARP-1 activity in aged hippocampus in contrast to a significant enhancement of PARP-1 activity in adults which may have important consequences for the physiology and pathology of the brain.

Keywords: PARP-1, poly(ADP-ribosyl)ation, brain, aging, oxidative stress, p53 protein, genotoxic stress

Poly(ADP-ribose) polymerase-1 (PARP-1, EC 2.4.2.30) is a highly conserved protein mainly localized in the nucleus. This about 113 kDa protein contains nuclear localization signal and DNA binding domain that recognizes DNA strand breaks. PARP-1 is activated by single and double DNA strand breaks, being the earliest and the most sensitive sensor of DNA damage (Malanga & Althaus, 2005). PARP-1 is responsible for the poly(ADP-ribosyl)ation of more than 40 nuclear chromatin-associated proteins, among them are p53, NF-κB, histones, AP-1, AP-2, topoisomerase I and II, and PARP itself. Automodified PARP-1 attracts the base excision repair complex facilitating removal of DNA damage (Masson et al., 1998; Pleschke et al., 2000; Strosznajder et al., 2005). A lack of PARP-1 activity causes hypersensitivity to genotoxic agents and elevates chromosomal abnormalities. Overactivation of PARP-1 by massive DNA breakage (Zhang et al.,

1994) leads to a depletion of its substrate βNAD⁺ and of ATP. These events are responsible for alteration of mitochondrial potential and may be involved in the release of apoptosis inducing factor (AIF) (Yu et al., 2002). Moreover, PARP-1 interacts with NF-κB and other transcription factors (Hassa & Hottiger, 1999; Oliver et al., 1999; Wesierska-Gadek & Schmid, 2001; Chiarugi & Moskovitz, 2003; Wesierska-Gadek et al., 2003; 2005). A growing body of evidence indicates the importance of PARP-1 in cell death during ischemia and neurodegenerative diseases (Strosznajder et al., 2003; 2005). The role of PARP-1 as a "guardian of the genome" has raised question about its significance in aging. Numerous publications have documented increased levels of damaged DNA and mutation frequency in aged organs (Mandavilli & Rao, 1996; Izzotti et al., 1999; Hamilton et al., 2001; Cabelof et al., 2002), thus it was of great interest to elucidate the influence of aging on PARP

Abbreviations: 3-AB, 3-aminobezamide; AIF, apoptosis-inducing factor; AP-1 and -2, activator protein-1 and –2; BSA, bovine serum albumin; DTT, DL-dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IR, immunoreactivity; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; βNAD⁺, beta-nicotinamide adenine dinucleotide; NF-κB, nuclear factor kappa B; NMDA, N-methyl-D-aspartate; P1, crude nuclear fraction; PMSF, phenylmethylsulfonyl fluoride; PARP-1, poly(ADP-ribose) polymerase-1; PBS-T, phosphate-buffered saline with 0.05% Tween-20; ROS, reactive oxygen species; RT-PCR, reverse transcription and polymerase chain reaction; SPF, specific pathogen free.

cedure in a total volume of 50 μl with 20 pmol of each primer. The primer sequences for p53 were 5'-TTCCCTCAATAAGCTGTTCTGCC-3' (forward) and 5'-TGCTCTCTTTGCACTCCCTGG-3' (reverse). As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was coamplified using primers 5'-TGAAGGTCGGAGTCAACGGATTT-GGT-3' (forward) and 5'-CATGTGGGCCATGAG-GTCCACCAC-3' (reverse). Each PCR cycle consisted of denaturation at 94°C for 1 min, annealing at 66°C for 1 min, and extension at 72°C for 1 min, followed by a final 7-min extension at 72°C. PCR amplification was carried out for 36 cycles using a Perkin-Elmer GeneAmp 2400 thermal cycler. After amplification, samples were separated on 2% agarose gel containing 200 μg/l ethidium bromide in 0.5× Tris/borate/EDTA buffer. The intensity of the p53 and GAPDH bands was estimated by densitometric analysis of the gel in UV light using a NucleoVision apparatus and GelExpert 4.0 software from NucleoTech.

Determination of PARP-1 activity in adult and aged hippocampus subjected to oxidative and genotoxic stress. For the determination of free radical-stimulated PARP-1 activity, the P1 nuclear fraction from hippocampus was preincubated with 25 μM $FeCl_2$ and 10 μM ascorbate for 1.5, 15 and 60 min. Then [adenine-^{14}C]NAD was added and the reaction was carried as was described above. For determination of the effect of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), the P1 nuclear fraction from hippocampus was pre-incubated for 15 min at 37°C and then incubated with 50 μM MNNG for 5 min. Then 200 μM βNAD$^+$ was added and the reaction was carried out for 1 min at 37°C. The reaction was terminated with denaturing sample buffer and the material was subjected to electrophoresis and transfer as described above. The membrane was blocked overnight in 5% non-fat dry milk and probed with 1:400 anti-poly(ADP-ribose) antibody (Alexis, clone# 10H) in 0.25% milk and then with 1:3000 secondary horseradish peroxidase-linked anti-rabbit IgG (Amersham) in milk. The bands were visualized with the ECL kit (Amersham) and analysed with a densitometer.

RESULTS AND DISCUSSION

Our present results demonstrate that in SPF animals PARP-1 activity is significantly elevated by 52% in aged hippocampus comparing to the adult one (Fig. 1). The activity of this enzyme in the cerebral cortex was also significantly enhanced by 64% while in cerebellum and striatum it was non-significantly elevated by 24% and 16%, respectively (Fig. 1). Our previous experiments demonstrated a significantly lower PARP-1 activity in aged *versus* adult hippocampus from non-SPF-category animals

(Strosznajder *et al.*, 2000a). We suggest that PARP-1 in non-SPF animals was subjected to excessive stress evoked by some kind of infection which markedly affected this enzyme in the hippocampus, leading in consequence to its covalent modification and inhibition. In the cerebral cortex and cerebellum the values were very similar to those observed previously (Strosznajder *et al.*, 2000a). Moreover, in the current study we also estimated the PARP-1 protein level. The immunoreactivity was unchanged in the aged hippocampus and cerebellum, non-significantly lowered in aged cerebral cortex and significantly down-regulated in the striatum of old rats (Fig. 2). These results indicate that the age related changes of PARP-1 occurr at the level of enzymatic activity, not on the protein expression level. Also Grube and Bürkle (1992) presented a lack of correlation between the amount of PARP protein and the life span with strong positive correlation between PARP activity and life span. They observed a higher specific enzyme activity in longer-lived species. In contrast, Messripour *et al.* (1994) demonstrated an enhancement of PARP activity and also PARP protein level in neurons and glia from aged rat brain. It is known from previous studies that PARP regulates/modulates p53 and NF-κB function (Malanga *et al.*, 1998; Chiarugi, 2002; Wesierska-Gadek *et al.*, 2003). It has been demonstrated that the activity of PARP-1 regulates expression of p53 (Agarwal *et al.*, 1997; Wang *et al.*, 1998). Our data indicated no changes of p53 expression in aged brain cortex comparing to the adult one and a non-significant enhancement of p53 mRNA level in the hippocampus and striatum (Fig. 3). However, significantly elevated p53 mRNA expression was found in aged cerebellum (Fig. 3). These results correlate with the higher PARP-1 activity in aged brain parts with the exception of brain cortex where p53 mRNA was not elevated. The data of Chung *et al.* (2000) presented higher immunoreactivity (IR) of p53 in the hippocampus and cerebel-
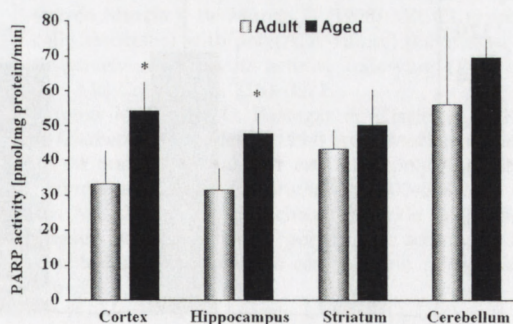

Figure 1. PARP-1 activity in nuclear fraction from different parts of adult and aged brains.
Bars represent values (means ±S.E.M.) from 3–5 animals. *$P < 0.05$ comparing to values from corresponding adult brain parts. Student's t-test.
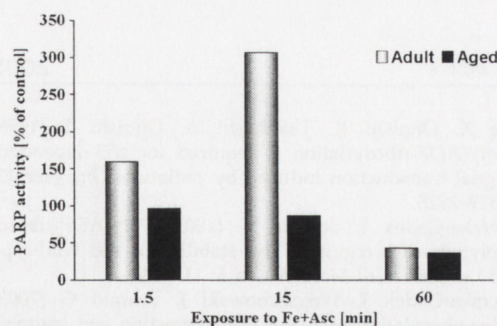
Figure 4. Effect of *in vitro* oxidative stress on PARP-1 activity in nuclear fraction from adult and aged hippocampus.
Nuclear fractions from adult and aged hippocampus were preincubated in the presence of 25 μM $FeCl_2$ and 10 μM ascorbic acid for 1.5, 15 and 60 min. Control values from non-treated adult and aged hippocampus were taken in each case as 100%. The values are means from one typical experiment carried out in triplicate. Pooled material from 3 adult and 2 aged animals was used.

data indicate that PARP-1 in aged hippocampus has a lower ability to respond to excessive oxidative or genotoxic stress. In consequence, the alteration of PARP-1 may have a protective effect against energy depletion but may also lead to a decreased efficiency of DNA repair machinery.

Acknowledgements

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Table 1. PARP activity in adult and aged hippocampus subjected to oxidative and genotoxic stress

Conditions	% of control	
	Adult	Aged
25 μM $FeCl_2$ + 10 μM Asc	157 ± 22*	111 ± 22
50 μM MNNG	258 ± 46*	83 ± 12

P1 nuclear fraction from hippocampus was incubated with $FeCl_2$ and ascorbate (Asc) for 1.5 min. Then enzymatic activity was measured using [adenine-^{14}C]NAD as described in the text. P1 nuclear fraction from hippocampus was incubated for 5 min with MNNG and subjected to Western blot for poly(ADP-ribose) as described in the text. Control values from non-treated adult and aged hippocampus were taken in each case as 100%. The values are means of 3–4 experiments ±S.E.M. *$P < 0.05$, Student's *t*-test.

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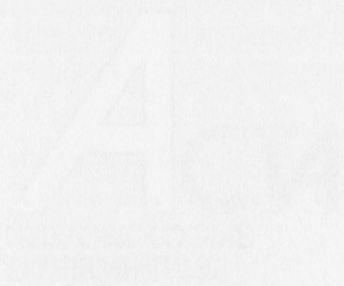
Omówione w dziale VI (str 34) dalsze kierunki badań zostały opublikowane w roku 2006 w Acta Neurobiol Exp 2006, 66: 15-22. Kopia pracy w załączeniu.

incubated for 1 min at 37°C and the reaction was stopped by adding 0.8 ml of ice-cold 25% trichloroacetic acid (TCA). Precipitate were collected on Whatman GF/B filters, washed three times with 5% TCA and left overnight for drying. The radioactivity was measured using scintillator counter LKB, Wallach 1409.

Differences in PARP activity were evaluated by unpaired Student's *t*-test.

Immunocytochemical electron microscopy studies

Before removal, the control, postischemic and treated by 3-AB brains were fixed by a transcardial perfusion with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (PBS), followed by a brief rinse with 0.9 % saline. Blocks of tissue were cut from stratum pyramidale of the CA1 sector of hippocampus, washed overnight in PBS, treated with 1% osmium tetroxide for 1 hour, dehydrated in the gradient of ethanol, and finally embedded in Epon. Ultrathin sections were treated according to post-embedding immunogold procedures. For double labeling the primary polyclonal antibody to Bcl-2 and monoclonal antibody to Bax (Santa Cruz Biotechnology sc-783; sc-20068) were diluted in PBS 1:20. After pre-treatment with 10 % hydrogen peroxide the antisera were applied to the tissue sections mounted on the formvar-coated nickel grids and incubated for 2 hours at 37°C. After washing, the sections were exposed for 1 h to secondary antibody coupled with 18 nm (Bcl-2) and 12 nm (Bax) gold particles, diluted 1:20 in PBS (Janssen, Beerse, Belgium).

For double labeling with monoclonal antibody to AIF and polyclonal antibody to Bcl-2 (Santa Cruz Biotechnology sc-13116; sc-783) antibodies were diluted 1:10 and the tissue sections were incubated overnight at 4°C and than after washing were exposed for 1 h to secondary antibody coupled with 12 nm (AIF) and 18 nm (Bcl-2) gold particles, diluted 1:10 in PBS. After immunolabeling the sections were washed with PBS and distilled water, dried and stained with uranyl acetate and lead citrate. All sections were examined in JEOL 1200EX electron microscope. For simple labeling the primary monoclonal antibody to AIF (Santa Cruz Biotechnology sc. 13116) was diluted in PBS 1:10 and incubated 3 h in 37°C. The secondary gold conjugated (20 nm) antibody diluted 1:20 in PBS

was applied as described above. The control staining was performed, where primary antibody was replaced by normal rat serum diluted 1:20 in PBS. The tissue sections were stained with 4.7 % uranyl acetate for 15 min and with lead citrate for 2 min. The sections were examined and photographed in a JEOL 1200EX electron microscope.

RESULTS

PARP activity in hippocampus after 3 min of transient global ischemia

The basal PARP activity value (mean ± SEM) from control sham operated animals was 22.35 ± 6.10. The PARP activity value from ischemic group (3 min ischemia, 4 days reperfusion) was 64.20 ± 4.57 which represented 287% of control value (the significance was at $P<0.01$). Intravenously administration of 3-AB directly after 3 min ischemia abolished the enhancement of PARP-1 activity observed at 4th days of reperfusion to the value close to the control.

Effect of brain ischemia on Bax and Bcl-2 immunolocalization in hippocampal area CA1. Protection with 3-AB

The brain sections were double labeled with anti-Bax and anti-Bcl-2 antibody, and visualized with gold particles of 12 and 18 diameters respectively. Figure 1 (A–D) illustrates subcellular localization of Bcl-2 and Bax. In control animal Bcl-2 is found close to the organelle membranes (mitochondria, rough endoplasmic reticulum (RER), Golgi complex, nuclear envelope) while Bax is generally found in cytoplasm (A). Three-minute ischemia and 4 days of reperfusion caused translocation of Bax from the cytoplasm to the membranes of mitochondria but did not change the localization of Bcl-2 (B). However, aggregates of Bcl-2, or a clusters consisting of Bax and Bcl-2 related proteins localized on mitochondrial, Golgi complex and on rough endoplasmatic reticulum (RER) membranes were also observed (B, C). Bax was also still visible in cytoplasm (C). 3-AB treatment causes evident overexpression of Bcl-2 protein in cytoplasm, but generally does not alter Bax expression. Bcl-2 protein aggregates are located not only on membranes of organelle, but also in cytoplasm and in nucleus of the neurons (D).

Effect of brain ischemia on AIF immunolocalization in hippocampal area CA1. Protection with 3-AB

The brain sections were labeled with anti-AIF antibody and visualized with gold particles 20 nm diameters. Figure 2 (A–D) illustrates subcellular localization of AIF. In control animal AIF is found only close to mitochondrial membranes (A). After ischemia AIF is preferentially translocated into the cytoplasm (B) and into the nucleus (C). Ischemia causes evident translo-
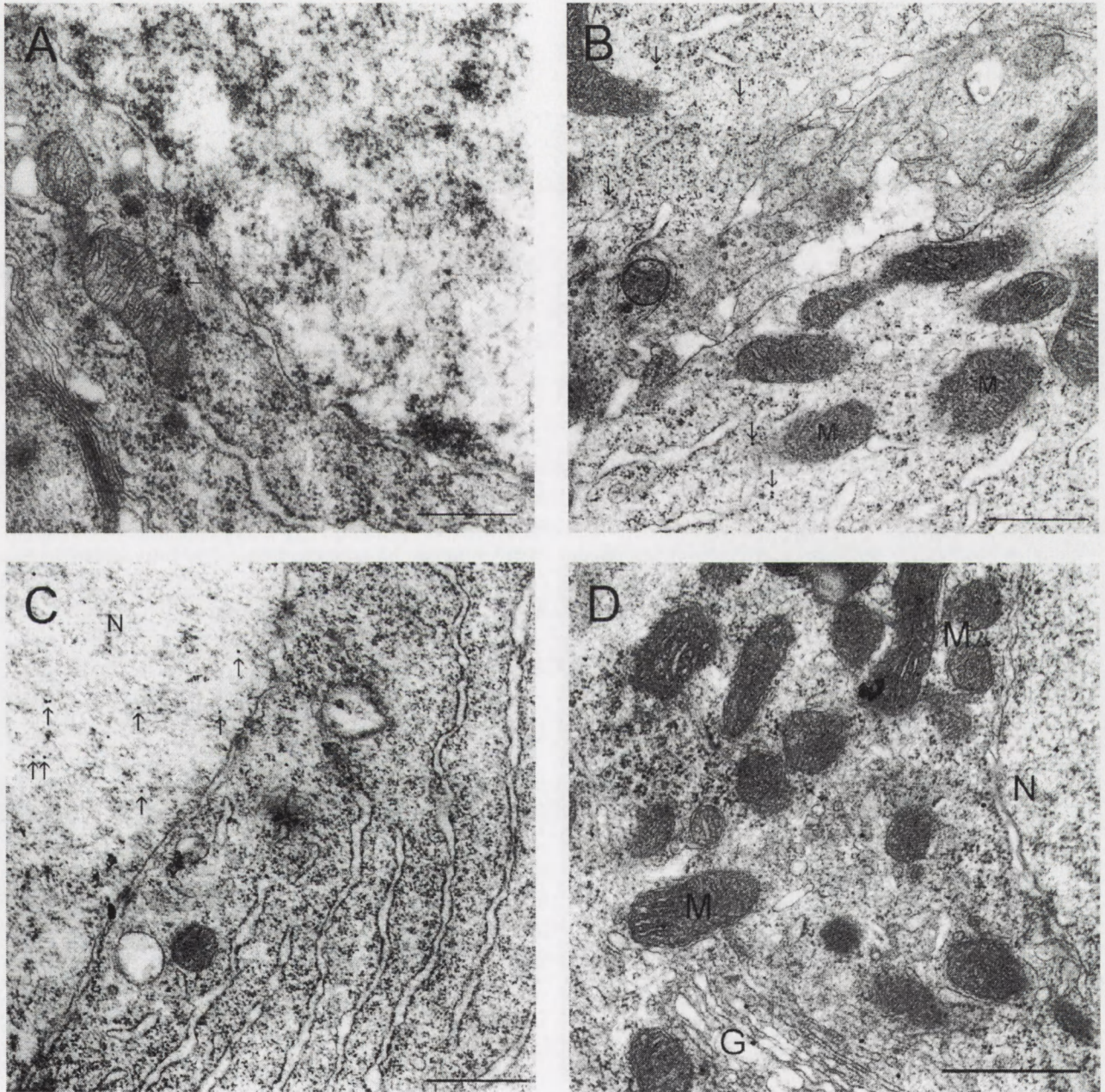

Fig. 2. Intracellular AIF localization in hippocampal area CA1 after ischemia-reperfusion injury. (A) AIF in control sham operated animal. Aggregate of gold particles (18 nm) representing AIF (arrow) on mitochondrial membrane are present. Bar is 500 nm. (B) Strong labeling for AIF (arrows) in cytoplasm after 3 min ischemia 4 days reperfusion. No labeling in ultrastructurally changed mitochondria (M). Bar is 500 nm. (C) AIF translocation (arrows) to the nucleus (N) after 3 min ischemia 4 days reperfusion. Bar is 500 nm. (D) Localization of AIF in ischemic CA1 after 3-AB treatment. On the membranes of unchanged mitochondria (M) and in the Golgi complex (G) labeling for AIF is present. Only a weak labeling in nucleus (N) or in cytoplasm is seen. Bar is 500 nm.

cation of AIF, especially from slightly ultrastructurally changed mitochondria to the nucleus (C). After 3-AB treatment AIF labeling is seen in the most unchanged mitochondria, on the Golgi complex (D). Only sporadically weak immunolabeling was observed in cytoplasm and nucleus (D).

Effect of brain ischemia on Bcl-2 and AIF immunolocalization in hippocampal area CA1. Protection with 3-AB

Subcellular localization of Bcl-2 and AIF were analyzed in brain sections using double labeling, where Bcl-2 and AIF were represented by 18 and 12 nm in diameter gold particles, respectively (Fig. 3A–D). In control animal Bcl-2 protein is located on organelle membranes, like Golgi complex, RER, nuclear envelope and mitochondria (A). AIF protein is found in connection with mitochondrial membranes and sporadically with RER (A). After ischemia massive translocation of AIF from mitochondria to the cytoplasm and to the nucleus was observed without altering of Bcl-2 expression (B). In 3-AB treated animal an increase of Bcl-2 labeling in cytoplasm and on membranes of intracellular organelles and in nucleus was found (C). Alternatively, a weak expression of AIF in cytoplasm and occasionally in nucleus was seen (C). In some ultrastructurally unchanged mitochondria the both AIF and Bcl-2 labeling was observed (D).

DISCUSSION

Our last studies Strosznajder and Walski (2004) and Strosznajder and coauthors (2005) demonstrated histological and ultrastructural indication of neuronal cell death and significant protection exerted by PARP inhibitor. In this study using immunocytochemical electron microscopy we have investigated the effect of 3-AB on localization and expression of two proapoptotic proteins Bax and AIF and anti-apoptotic Bcl-2 protein. Our data indicate that Bcl-2 overexpression is able to prevent cell death probably through the interacting with Bax and AIF. There are several reports indicating that overexpression of Bcl-2 is able to inhibit neuronal death in various experimental paradigms both *in vitro* and *in vivo* (Allsopp et al. 1993, Dubois-Dauphin et al. 1994, Martinou et al. 1994). Upregulation of Bax and decrease in Bcl-2 was

demonstrated after focal ischemia in the rat brain (Gillardon et al. 1996). It was also reported that Bax expression precedes DNA fragmentation in the CA1 region of gerbil hippocampus. Other studies also presented changes in Bcl-2 family expression after focal and global ischemia (Shimazaki et al. 1994, Honkaniemi et al. 1996). Also the study of Linnik and coauthors (1995) showed that in focal cerebral ischemia, Bcl-2 overexpression in the brain protects neurons from ischemic brain damage *in vivo*. Also Sorenson (2004) using middle cerebral artery occlusion model found that, following 7 days of permanent focal cerebral artery occlusion, there was a 43% reduction of ischemic volume in transgenic mice (which neurons overexpress human Bcl-2) compared with wild-type animals. Modulation of Bcl-2 and Bax expression after MCA occlusion may also be mediated by tumor suppressor p53 which is induced within ischemic brain regions and which regulates Bcl-2 and Bax gene expression in other models of cells death (Miyashita et al. 1994). On the other hand the mitochondrial proteins such as: cytochrome c, caspases, Omi/HtrA 2 or endonuclease G may be affected by Bcl-2 overexpression as it was demonstrated previously (Daugas et al. 2000, Mathiasen and Jaattela 2002, Zhao et al. 2003). Our data demonstrate nuclear AIF translocation from mitochondria to the nucleus suggesting that AIF apoptotic pathway in global ischemia is activated. Recently it was established that nuclear AIF translocation depends upon the activity of poly(ADP–ribose) polymerase 1 (PARP-1) during NMDA–induced neuronal death (Yu et al. 2002). Our data showed that 3-AB protected neurons against AIF dependent apoptosis. Moreover, 3-AB treatment induced overexpression of Bcl-2 and significantly improved ultrastructural morphology of the neuron.

CONCLUSION

We suggest that overexpression of anti-apoptotic protein Bcl-2 may block nuclear AIF translocation and improve CA1 hippocampal neurons survival after global ischemia.