A szenzoros neuron effektor működésének vizsgálata experimentális neuropátiában

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1 TARTALOMJEGYZÉK

2 RÖVIDÍTÉSEK JEGYZÉKE

B2m	béta 2- mikroglobulin
CGRP	calcitonin gén-rokon peptid
CGRPR	calcitonin gén-rokon peptid receptor
Ct	a küszöb fluoreszcencia eléréséhez szükséges ciklusszám
DM	diabetes mellitus
DNS	dezoxi-ribonukleinsav
DRG	hátsó gyöki ganglionok
ENG	elektroneurográf
EPO	erytroprotein
FPG	éhgyomri vércukor koncentráció
FPI	éhgyomri inzulin koncentráció
HOMA	homeasztatikus modell kiértékelése
HOMA-IR	perifériális inzulin rezisztencia
HOMA-%B	B-sejt funkció vizsgálata
i.c	intracelluláris
i.p.	intraperitoneális
L-NAME	NG- nitro- L-arginin metil észter
n. saphenus	nervus saphenus
n.vagus	nervus vagus
NaCl	Nátrium-Klorid
NANC	non-adrenerg, non-kolinerg
NK1	Neurokinin 1 receptor
NK2	Neurokinin 2 receptor
PPT-A	Preprotachykinin-A
QRT-PCR	quantitative real-time polymerase chain reaction
RIA	radioimmunoassay
RNS	ribonukleinsav
S.D.	standard deviáció
S.E.M.	standard error of means
SOD	szuper-oxid dizmutáz
SOM	szomatosztatin

SP	P-anyag
SRIF I, II	szomatosztatin receptor alcsalád I, II
SSTR1, 2, 3, 4, 5	szomatosztatin receptor 1., 2., 3., 4., 5. altípusa
STZ	streptozotocin
TI	téringerlés indukálta
TTX	tetrodototoxin

1. Táblázat: Rövidítések jegyzéke

3 ÁLTALÁNOS BEVEZETÉS

A szenzoros neuron farmakológiája néhány évvel ezelőtt még hiányzott a farmakológia tárgyú tankönyvekből. Az elmúlt fél évszázad kutatásai azonban jelentős eredményeket hoztak e területen és lassan körvonalazódni látszik, hogy hogyan és milyen célból tudjuk majd befolyásolni az érző neuronok működését. Jelenleg még nem rendelkezünk szelektíven a szenzoros neuronon ható fájdalomcsillapítóval, de ennek megjelenése nem sokáig kell várasson magára. Fontos megemlíteni azt is, hogy a szenzoros neuron nem csak arra hivatottak, hogy a periféria felől a centrumba juttassák az információt (orthodrómos vezetés), hanem speciális effektor funkcióval is rendelkeznek (antidrómos vezetés), mivel az ingerületre belőlük felszabaduló neuropeptidek parakrín (de egyes esetekben endokrín) hatásokat hoz létre. Ezen effektor hatásnak szerepe lehet a gyulladásos folyamatok mechanizmusában valamint a mikrocirkuláció szabályozásában. E rendszer kutatását jelentősen segítette, hogy David Julius munkacsoportjának sikerült a szenzoros neuronra jellemző capsaicin/vanilloid receptor klónjait előállítani (VR1 receptor) (Caterina és mtsai, 1997). A capsaicin receptorokon való hatásának első leírása tisztán farmakológiai eszközökkel szerkezet-hatás összefüggések vizsgálatának következményeként került sor (Szolcsányi és mtsai, 1975). A receptor klónozása ezeket a vizsgálatokat évtizedek elmúltával erősítették meg. Napjainkban számos gyógyszergyár (Novartis, SKB, Procter and Gamble stb.) intenzív kutatásokat folytat szenzoros neuronon szelektíven ható vegyületek kifejlesztése érdekében.

A szenzoros neuronok szerepet játszanak néhány betegség pathomechanizmusában vagy a betegség lefolyása a szenzoros neuronok funkcióját befolyásolja. Kísérleteink éppen ebből a szemszögből vizsgálják a szenzoros neuron effektor működését. A daganat kemoterápia során sokszor merül fel a neurotoxicitás problémája és hasonló módon a cukorbetegség szövődményei közt is sok esetben problémát jelent mind a beteg, mind az orvos számára a hosszú lefolyás során fellépő neuropathia. Természetes, hogy ezek a behatások nem csak a szenzoros neuront érintik, hanem hatással vannak pl. a vegetatív neuronokra is. Mivel várható, hogy az érző neuronon kifejtett neurotoxikus mellékhatás vizsgálata számos új eredménnyel kecsegtet, vizsgálatainkat ezen neuroncsoport működésének vizsgálatára fókuszáltuk.

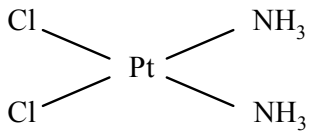
3.1 Experimentális neuropátia, mint vizsgálati módszer

Az idegkárosodás (neuropátia) számos betegség kisérő tünete, önálló kórképként is előfordul, ill. jelentkezhet gyógyszer mellékhatásként, valamint terápiás szövődményként. Szokás szenzoros, autonóm, illetve motoros neuropátiáról beszélni, aszerint, hogy a funkciózavar mely idegeken domináns. Munkánkban a szenzoros neuropátia, illetve a szenzoros idegkárosodás funkcionális következményeit vizsgáltuk experimentális körülmények között, elsősorban olyan állatkísérletes modellekben, amelyekből levonható következtetéseknek közvetlen klinikai vonatkozásai vannak. Ezért esett a választás a daganatos betegségek terápiájában gyakran használt cisplatin indukálta idegkárosítás experimentális vizsgálatára. A cisplatin választásához az is hozzájárult, hogy e farmakon indukálta szenzoros neuropátia, mely a cukorbetegség kapcsán szövődményként is jelentkezik, jól modellezhető ezzel a farmakonnal.

3.2 A cisplatin felfedezése, hatásmechanizmusa, terápiás alkalmazása

Az 1960-as években észrevették, hogy a platina gátolni képes az E. coli osztódását (Rosenberg és mtsai 1965). Bár e megfigyelés után számos platina tartalmú vegyületet állítottak elő (carboplatin, oxoplatin, cisplatin), közülük az egyik leggyakrabban használt szer a cisplatin maradt. A mai napig számos gyógyszergyár gyártja. Kísérleteinket a TEVA-BIOGAL GYÓGYSZERGYÁRTÓ RT. által forgalmazott „cisplatin-TEVA”-val végeztük. A cisplatin egy platinaszármazék, a vegyület két amino- és két klorid csoportot tartalmaz (cis-diamindikloroplatinum (II). Elsősorban hererák és petefészekrák gyógyítására használják, alkalmazása során remisszió, esetleg teljes gyógyulás érhető el. Használják még hörgő-, méhnyak-, prosztata- és urothelialis rákok, valamint kissejtes tüdőrák kezelésében sugárterápiával kombinálva. A cisplatin a sejtekbe diffúzióval jut be. Intracellulárisan (ic.) a klorid szint alacsony, ezért a molekula 2 klorid ionja lehasad. A kloridionok helyére két vízmolekula kötődik. A pozítívan töltött vegyület akkumulálódik a sejtben, és kölcsönhatásba lép nukleofil komplexekkel, pl. tiolokkal, fehérjékkel, nukleinsavakkal. (Gonzalez és mtsai 2001). A cisplatin az intakt DNS molekulában elsősorban a guanin és az adenin purin bázisok 7-es N-atomjához kötődik. A kialakult kötések nagy része két olyan guanin molekulát kapcsol össze, amelyek a DNS azonos szálán találhatóak. A cisplatin primer hatását a DNS

térszerkezetének módosításával fejti ki (Welters és mtsai 1999). A DNS-en kívül még képes kötődni plazma fehérjékhez, jelentősen károsítja a vér-agy gátat és a környéki idegrendszert (Quasthoff és Hartung 2002). Terápiás potenciálját nagymértékben csökkentik mellékhatásai. A kezelés során kialakulhat myelo-, nephro-, oto- és neurotoxicitás (Fillastre és Raguenez-Viotte 1989; Ozols és Young 1985; Stewart és mtsai 1987).



1. ábra: Acisplatin szerkezeti képlete

3.3 A cisplatin okozta myelotoxicitás

Cisplatin kezelés hatására enyhe myeloszupresszió alakul ki, mindhárom vérképző sejtvonalat érinti (McKeage 1995). A myeloszuppresszió hatására kialakuló anémia, leukopénia és trombocytopénia dózisfüggő. A cisplatin indukálta anémia jól ismert mellékhatása a kezelésnek, a páciensek mintegy 9 ± 4%-ban fordul elő. A kezelés hatására kialakult anémia a páciensek életminőségét rontja. Anémia hatására gyengeség, szívizom iszkémia, ill. egyéb szervek vérellátási zavarai alakulnak ki, munkaképességük csökken. A tünetek megszüntetésére súlyos fokú és hirtelen kialakuló, panaszokat okozó anemizálódás esetén terápiás beavatkozásként vértranszfúzió adása javasolt, annak minden előnyével és potenciális veszélyeivel együtt. Állatmodellekben és klinikai kísérletekben az exogén rekombináns humán erytroprotein (EPO) kezelés megszünteti a cisplatin indukálta anémiás tüneteket. Kettősvak klinikai vizsgálatok során bizonyítást nyert, hogy az EPO használata mellett a szükséges vér transzfúzió mennyiségét csökkenteni lehet (Pivot és mtsai 2000). Ha azok a rákos betegek, akik kemoterápiás kezelésen mentek keresztül, alternatív kezelésként EPO-t kapnának, akkor megelőzhetnénk az anémia kialakulását. Azonban profilaktikus kezelésként az EPO túlságosan drága, így csak akkor alkalmazható, ha a pácienseknél anémiára hajlamosító jeleket találnak (Pivot és mtsai 2000). A vérképző rendszer érintettsége miatt az anémia mellett kialakulhat még a vérlemezkéket és fehérvérsejteket érintő érési zavar trombocitopénia és leukopénia formájában. Ezek mértékét az amifostin időfüggő módon

csökkenti (Asna és mtsai 2005). A cisplatin kezelés 100mg/m^2 dózisban szignifikánsan képes csökkenteni a leukociták számát (Anderson és mtsai 1988). Cisplatinnal kezelt petefészek-daganatos betegekben, a kezelés után növekszik a szekunder leukémia kialakulásának kockázata, míg a csak sugárterápiával kezelt betegekben nem (Travis és mtsai 1999).

3.4 A cisplatin okozta nefrotoxicitás

Mivel a cisplatin elsősorban a vesén keresztül választódik ki, megfigyelhető a vesekéregben az akkumulációja. A kezelés után 3-5 nappal degeneratív változások alakulnak ki a proximális tubulusban: citoplazmatikus vakuolizáció, tubuláris dilatáció, piknotikus és hidropikus degeneráció. A vese vérátáramlása már nagyon kis dózis után lecsökken. Gyakran előfordul hipomagnéziémia, hipokalcémia és hipokalémia. A cisplatin indukálta nephrotoxicitás pontos mechanizmusa egyelőre ismeretlen. A cisplatin biotranszformációja fontos szerepet játszhat a folyamatban. Először a szulfilhidril csoportok mennyisége csökken a vesében, mely valószínűleg reaktív metabolitokat képez. A betegeknél prehidrálással, vagy kalcium blokkolók adásával szignifikánsan csökkenthetjük a cisplatin nefrotoxikus hatását. A klinikumban ezért a gyógyszert infúzión keresztül, lassan adagoljuk (1mg/kg/h). A nefrotoxicitás mértéke hidratálással, valamint ozmotikusan aktív anyag, a mannitol adásával csökkenthető. A cisplatint (Cisplatin-TEVA 10 mg/20 ml) a klinikumban i.v. adják, a következő adagolási séma szerint: öt napon keresztül naponta 20-50 mg/m^2, vagy háromhetente egyszer 100mg/m^2 4 hetente egyszer monoterápiában. A cisplatin oldatokat 2l,5 %-os glükóz oldatot és esetleg 37.5g mannitot tartalmazó 0.3 vagy 0.45 %-os nátriumklorid infúziós oldattal kell hígítani és 6-8 órás infúzióban beadni. A kezelés során ügyelni kell, hogy a páciens megfelelő mennyiségű folyadék terápiában részesüljön (pl. 1 – 2l Ringer – laktát infúzió) és az infúziót követő 24 órán át biztosítani kell a megfelelő folyadékbevitelt és diurézist. Kísérleteink során a cisplatin nephfrotoxikus hatását szintén mannitol adásával védtük ki.

3.5 A cisplatin okozta ototoxicitás

A cisplatin ototoxicitása dózisfüggő és kumulatív, gyakran krónikus szenzoros halláskárosodást okoz. Kezdetben a magas frekvenciájú hangok, majd ezt követően az alacsony frekvenciájú hangok hallása is károsodik (Schweitzer 1993). A cohlea külső szőrsejteiben cisplatin kezelés hatására feltehetőleg $O_2^{\cdot-}$ szabadgyökök képződnek NADPH

oxidáz vagy egyéb enzim segítségével. Fenton reakción keresztül -OH képződhet, mely később a sejtmembrán többszörösen telítetlen zsírsavaival kapcsolatba lépve toxikus aldehideket képezhet. Ilyen melléktermék pl. a 4-hidroxi-nonenal, mely sejthalált képes okozni. $O_2^{-\cdot}$ aktiválhatja az indukálható nitrogén-oxid szintáz (iNOS) enzimet, mely nitrogén-oxidot (NO) termel. Az $O_2^{-\cdot}$-ből a nitrogén-oxidon keresztül peroxinitrit képződik, mely később reagál a fehérjék tirozin aminosavaival, nitrotirozinná alakítja azokat. Ezek a toxikus intermedierek fokozhatják citokróm-C kiáramlását a mitokondriumokból. A citokróm-C kiáramlása aktiválja a kaszpáz rendszert (kaszpáz 3-t és 9-t), mely enzimek apoptózist okoznak (Rybak és Whitworth 2005).

3.6 A szenzoros neuron duális funkciójának károsodása cisplatin neurotoxicitás következtében

A cisplatin neurotoxicitása közismert. Cisplatin kezelés után általában egy hónappal jelennek meg a szenzoros neuropátiára jellemző tünetek (paresztézia, ataxia, Lhermitte-jel) (Grunberg és mtsai 1989). Barajon neuromorfológiai tanulmányai szerint a cisplatin-indukálta változások alapja az, hogy a szenzoros neuropeptidek calcitonin-génrokon peptid, P-anyag, szomatosztatin (CGRP, SP, és SOM) hátsó gyöki ganglionok (dorsal root ganglion (DRG)) szómájában akkumulálódnak. Az akkumuláció során a ganglion sejtjeinek hisztokémiai elváltozásai arányosak perifériás rostok károsodásának mértékével (Barajon és mtsai 1996). Krónikus cisplatin kezelés hatására a plazma idegnövekedési faktor (Nerve Growth Faktor; NGF) szintje csökken (Cavaletti és mtsai 2002). Az NGF befolyásolja a preprotachykinin-A transzkripcióján keresztül az SP termelődését. Az NGF szintén szabályozza a CGRP transzkripcióját (Diemel és mtsai 1992). A cisplatin kezelés indukálta szenzoros-effektor funkciók károsodását a felszabaduló szenzoros neuropeptidek hatásain keresztül vizsgáltuk.

3.7 Diabetes mellitus

Az I-es típusú diabetes mellitus (DM) inzulin hiány miatt alakul ki, és megfelelő kezelés nélkül hiperglikémiához, polidipsziához, poliuriához, súlyvesztéshez valamint súlyos szervi szövődményekhez vezet. A nem megfelelően kezelt DM szövödményei pl. a fájdalmas perifériás idegkárosodás (Max és mtsai 1992), a gyulladás indukálta válaszok gyengülése, a vaszkuláris permeabilitás megváltozása (Ahmad és mtsai 2005), mely számos

kardiovaszkuláris megbetegedést okoz. Ilyenek lehetnek pl. a stroke és a hipertenzió (Ferrari és Weidmann 1990). Streptozotocin (STZ) indukálta diabétesz patkányban az I. típusú cukorbetegséghez hasonló tüneteket okoz, beleértve a krónikus fájdalmat, megnövekedett vaszkuláris permeabilitást, és a gyulladást (Courteix és mtsai 1993; Tomlinson és mtsai 1992).

3.8 Diabétesz és az asztma kapcsolata

Az egyes típusú cukorbetegség és az asztma egyszerre ritkán fordul elő (Belmonte és mtsai 1998; Casaco és mtsai 1989; Helander 1958). Az irodalom szerint a vagus reflexek központi szerepet játszanak a bronchiális hiperreaktivitás kialakulásában, melyet az epithelium károsodása okoz (Barnes 1990). A hiperreaktivitás kialakulásával a légutak érzékenysége spazmogénekkel szemben nem változik meg *in vivo*. Mindazonáltal a hiperreaktivitás némely formája a n. vagus átmetszésével megakadályozható (Sanjar és mtsai 1990). Mivel a nervus vagus rostjainak 90%-a szenzoros rost, nem meglepő, hogy capsaicin előkezeléssel a légutak hiperreaktivitásának némely formája megelőzhető (Perretti és Manzini 1993). A szenzoros neuropeptidek infúziója viszont légúti hiperreaktivitást okoz tengerimalacban (Chapman és mtsai 1993). Korábbi kísérletek azt mutatták, hogy diabéteszben téringerlés hatására patkány tracheából a szenzoros neuropeptidek (SP, CGRP, SOM) felszabadulása szignifikánsan csökken (Nemeth és mtsai 1999a). Azonos körülmények között megfigyelt két jelenség, azaz a téringerlés indukálta (TI) szenzoros neuropeptid felszabadulás csökkenés, valamint a bronchusgyűrű-kontrakció gyengülés alapján feltételeztük, hogy a jelenségek között ok-okozati kapcsolat van, azaz a téringerlés hatására csökkent szenzoros neuropeptid felszabadulás okozza a TI bronchokonstrikció gyengülést.

3.9 Cisplatin indukálta szenzoros neuropátia és a diabétesz során létrejövő szenzoros neuropátia közös vonásai

Diabétesz hatására a polyol anyagcsereút károsodik, a szorbitol és fruktóz felhalmozodása endoneurális ödémát és idegi iszkémiát okoz (Bravi és mtsai 1997). A gerincvelő lumbális L4-L6-os szakaszában a DRG sejtekben a preproCGRP, preproSP és a preproSOM mRNSeinek transzkripciója szignifikánsan csökken (Rittenhouse és mtsai 1996). Végső soron mind a diabétesz, mind a cisplatin indukálta neuropátiában csökken az axonterminálisokból felszabaduló szenzoros neuropeptid mennyiség. A szenzoros neuropeptid

felszabadulása –ugyan különböző okok miatt– mind a cisplatin indukálta, mind a diabétesz során létrejövő neuropátiában csökken, ezért indokolt mindkét modellben megvizsgálni a szenzoros effektor funkcióváltozásokat.

3.10 Az experimentális szenzoros neuropátia vizsgálatához alkalmazott módszerek

A cisplatin és a diabétesz indukálta szenzoros neuropátia vizsgálatát legkönnyebben izolált bronchuspreparátumokon lehet vizsgálni. Ismert ugyanis, hogy a bronchus preparátumok gazdagon innerváltak CGRP, SP, neurokinin-A és SOM tartalmú myelinizálatlan afferens rostokkal, melyek a felszín közelében, a mukóza alatt húzódnak (Lundberg és mtsai 1983; Lundberg és mtsai 1984). Ezek a rostok a nervus vagus ganglion nodusum, ganglion superior és a nucleus dorsalis nervi vagi-ból erednek (Springall és mtsai 1987). A bronchomotilitás szabályozásában betöltött szerepük miatt ezeknek a rostoknak a hagyományos érző funkciójuk mellett effektor funkciójuk is van. Ismert továbbá az is, hogy a bronchusokban a felszín közelében, a mukóza alatti szövetekben expresszálódnak a szenzoros neuropeptidek receptorai is. Ilyen neuropeptid receptorok az alfa- és a béta- CGRP receptor, neurokinin-1, neurokinin-2, és a nagy mennyiségben expresszálódó 4-es valamint a kis mennyiségben expresszálódó 1-es típusú szomatosztatin receptor. (CGRPR, NK_1, NK_2, SSTR4, SSTR1) (Helyes és mtsai 2003a). E rostok különböző stimulusok, mint pl. az extracelluláris K^+ ion koncentrációjának növekedése, a szöveti pH csökkenése, vagy elektromos impulzusok hatására környezetükbe ürítik neurotranszmittereiket (Szolcsanyi 1996), melyek a bronchomotilitást is befolyásolni képesek. A neurotranszmitterek felszabadulásának változását radioimmunoassay (RIA) módszerrel, a bronchomotilitás vizsgálatokat elektromos impulzusokkal vagy kémiai vegyületekkel kiváltott bronchomotilitásra kifejtett hatások tanulmányozásával vizsgáltuk. A receptorok mRNS-ei tüdőből könnyen, és nagy mennyiségben izolálhatóak, általuk vizsgálhatóak a neuropeptid receptorok transzkripciós mintázata quantitative real-time polymerase chain reaction (QRT – PCR) segítségével.

3.11 A szenzoros neuropeptidek és receptoraik

3.11.1 *Calcitonin gén-rokon peptid (CGRP)*

A CGRP, - mely a szerveket innerváló szenzoros idegrostokból szabadulhat fel -, a légző rendszerben mind fiziológiai, mind patofiziológiai körülmények között fontos szerepet játszik. Az elmúlt években számos tanulmány írta le szerepét a broncho- és vazoregulációban, valamint a neurogén gyulladás folyamatában (Lundberg 1996; Lundberg és mtsai 1985). A CGRP-t expresszáló rostok a nucl. trigeminus, a ganglion nodosum és a hátsó gyöki ganglionokból erednek. A CGRP 37 aminosavból álló peptid, a calcitonin pre-mRNS-ből alternatív splicing során alakul ki. A receptorának két izoformája van, (alfa-CGRPR és béta-CGRPR) melyek mindössze 3 aminosavban különböznek egymástól. A receptor az úgynevezett G-fehérjéhez kapcsolt receptorcsalád tagja. Mint ahogy más neuropeptid, a CGRP is felszabadulhat primer afferens rostokból fájdalmas inger, elektromos vagy kémiai inger hatására. Az afferens rostokban, mint pl. a légző rendszerben a CGRP és az SP együtt tárolódik (Springer és mtsai 2003).

3.11.2 *P-anyag (SP)*

Számos szervben, mint pl. a bőrben és a légutakban a capsaicin-szenzitív rostokból ingerlés hatására SP és NKA szabadul fel. E neurotranszmitterek a gyulladás kialakulásában játszanak szerepet (Maggi 1995b; Holzer 1988). A gyulladással járó kórképekben a stabil tachykinin antagonistáknak terápiás jelentősége lehet. Az SP származhat mind az érzőideg végződésekből, mind a gyulladás folyamatában részt vevő sejtekből. A SP kisebb mértékben vazodilatációt is okoz, de fő hatása a plazma protein extravazáció. Az SP hatására a granulociták és az endothel sejtek adhéziós molekulákat termelnek (Maggi 1995a; Nakagawa és mtsai 1995). Az SP a légutakban az NK_1, NK_2 receptorokon keresztül fejti ki hatását.

3.11.3 *Szomatosztatin (SOM)*

A SOM 14 és 28 aminosavat tartalmazó formái mind a központi, mind a környéki idegrendszerben megtalálhatóak. A pankreász D-sejtjei, valamint a szenzoros, és szimpatikus idegsejtek termelik. A natív neuropeptid plazma felezési ideje kevesebb, mint 3 perc (Helyes

és mtsai 2003b). A SOM receptorok 5 altípusát klónozták. A receptorok a G-fehérjéhez kapcsolt receptorok családjába tartozik, melyek az adenilát-cikláz enzimet gátolják, így csökkentik a Ca^{2+}-csatornák vezetőképességét, aktiválják a K^{+}-csatornát. Két nagy csoportra oszthatóak: az SSTR1 és 4 a szomatosztatin receptor II-s (SRIFII)-s család, az SSTR2, 3 és 5. pedig a szomatosztatin receptor I-s (SRIFI)-es receptorcsalád tagjai (Hoyer és mtsai 1995). Patkány tüdőben elsősorban az SSTR4 és kisebb mennyiségben az SSTR1 receptorok expresszálódnak. A SOM gyulladásgátló hatását valószínűleg az SSTR4-en keresztül fejti ki. Számos endogén tumor expresszál SSTR-t, melyek gyógyítására szintetikus SOM molekulákat, mint pl. octreotidot használnak. Az octreotid elsősorban az SRIFI receptorcsaládra hat, míg a SRIFII-es család tagjaihoz kisebb affinitással kötődik (Helyes és mtsai 2003b).

4 CÉLKITŰZÉSEK

1. Befolyásolja-e a cisplatin indukálta szenzoros neuropátia
    a. a téringerlés következtében létrejövő bronchomotilitást?
    b. a tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek mennyiségét?
    c. a plazma neuropeptideinek (VIP, SP, CGRP, SOM) szintjét?
    d. a neuropeptid receptorok (NK1, NK2, CGRPR, SSTR4) mRNS-ének transzkripcióját?
2. A diabétesz következményeként létrejövő szenzoros neuropátiában kimutatható-e összefüggés a TI bronchomotilitás változás és a tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek csökkenése között?
    a. Befolyásolja-e az STZ-kezelés a plazma SOM szintet?
    b. Megváltoztatják-e diabéteszben a TI bronchokonstrikciós válaszokat az exogén szenzoros neuroropeptidek (CGRP, SP és SOM)?
    c. Megváltozik-e az antigén indukálta bronchokonstrikció antigénnel szenzitizált diabéteszes tengerimalacban?

5 MÓDSZEREK

5.1 Etikai engedélyek

A szükséges kísérleteket az Európai Unió laboratóriumi állatok használatáról és gondozásáról szóló, jelenleg érvényben lévő jogszabályok betartásával végeztük el. A kísérleti protokollt mind a Debreceni Egyetem, mind a Pécsi Tudományegyetem Etikai Bizottsága elfogadta.

5.2 A felhasznált anyagok és eszközök

5.2.1 *Felhasznált vegyszerek*

Anyag neve	Gyártó	Telephely
[Tyr1]- szomatosztatin	Bachem	Bachem
125-Ival jelzett tracerek	Farmakológiai Int.	Pécs, PTE
Atropin	Sigma-Aldrich KFT.	Budapest
B2M, CGRPR, NK_1, NK_2, SSTR4 Primerek	Sigma-Aldrich KFT.	Budapest
Capsaicin	Fluka	Buchs
CGRP	Sigma-Aldrich KFT.	Budapest
CGRP, szom antiszérum	Dr. Görcs T. ajándéka	SOTE, Budapest
Cisplatin	TEVA- Biogal	Debrecen
EDTA	Sigma-Aldrich KFT.	Budapest
Guanetidine	Sigma-Aldrich KFT.	Budapest
Inzulin RIA KIT	Izinta KFT	Budapest
Inzulin inplantatum	Linplant	Dánia
Ovalbumin	Sigma-Aldrich KFT.	Budapest
LightCycler RNA Master SYBR Green I Kit	Rosche Applied Science	Budapest
L-NAME	Sigma-Aldrich KFT.	Budapest
Metanol	Carlo Erba	Limito

Na_2HPO_4	Reanal	Budapest
NaCl	Fluka	Budapest
Piperin	Fluka	Budapest
Polipropilén csövek (RIA)	Merck	Darmstadt
Rneasy Mini Kit (Quiagene Inc.)	Kasztel Med. KFT (forgalmazó)	Budapest
SP	Sigma-Aldrich KFT.	Budapest
SP antiszérum	Prof. G. J. Dockray ajándéka	
SP-tracer	Bachem	Budapest
STZ (Zanosar)	Upjohn	Kalamazoo
Trasylol	Bayer	Budapest
TTX	Sigma-Aldrich KFT.	Budapest
Tween 80	Reanal	Budapest
Tyr-α-CGRP	Bachem	Budapest

2. táblázat: Felhasznált anyagok és vegyszerek

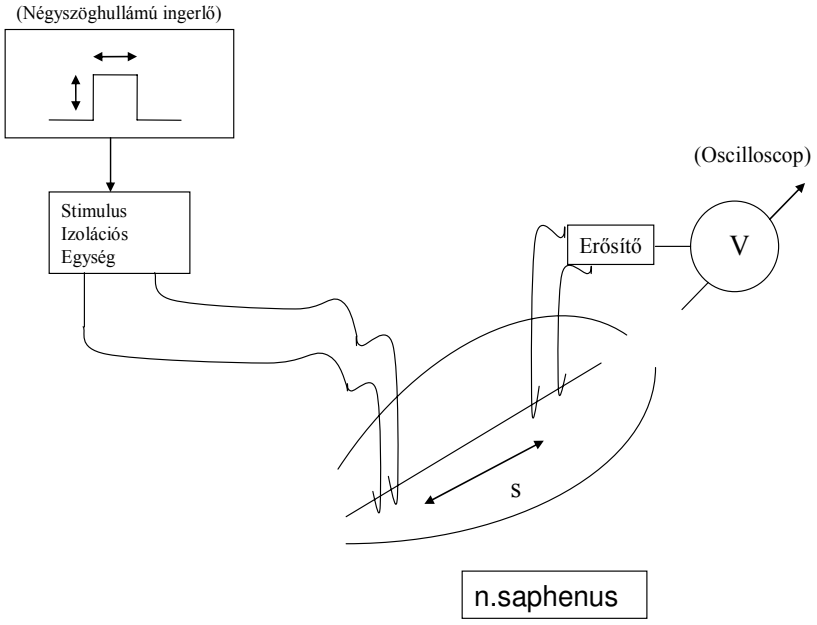
5.2.2 *Felhasznált eszközök*

Eszközök	Cég neve	Telephely
CWB – 20 water thermostat with circulatory pump	Experimetria Kft.	Budapest
TSZ – 04 multi chamber modular tissue bath system	Experimetria Kft.	Budapest
CRS – SG bridge amplifier for force measurement	Experimetria Kft.	Budapest
Accu Check	Roche- Diagnostic	Budapest
LightCycler 1.5r	Roche (Magyarország) Kft.	Budapest
Nanodrop	BIO-SCIENCE Ltd.	Budapest

3. táblázat: Felhasznált eszközök

5.3 Az ingerületvezetési sebesség (Nerve Conduction Velocity, NCV) mérése

A vizsgálatokat a szenzoros neuropátia kizárása vagy megerősítése miatt végeztük el. A kísérlethez használt állatokat tiopentantállal (Trapanal® 30 mg/kg i.p.) elaltattunk és pipecuromium bromiddal (Arduan® 0.3 mg/kg i.v.) harántcsíkolt izomrelaxációt hoztunk létre. Az állatokat a tracheába helyezett kanülön keresztül mesterségesen lélegeztettük. A bal oldali n. saphenust kipreparáltuk és megtisztítottuk a környező zsírszövettől és a kötőszövettől (Nemeth és mtsai 1999b). Két elektróda párt helyeztünk fel az így megtisztított idegre, egymástól kb. 2 cm távolságra (2. ábra). Ezután a proximális elektródán keresztül konstans négyszögimpulzusokkal (500 μs) ingereltünk és a disztális elektródáról elvezettük az elektromos stimulusok által kiváltott summációs akciós potenciálokat. A kiváltott stimulus és a szummációs akciós potenciál megjelenése között eltelt idő alapján, az elektródapár egymástól mért távolságának ismeretében az ingerületvezetési sebesség meghatározható volt (Janig és Lisney 1989).



2. ábra Ingerületvezetés sebesség mérése

5.4 Az izometriás feszülés változásának mérése

Az állatok kivéreztetése után eltávolítottuk a tracheát a tüdőkkel együtt, majd 3 mm vastagságú gyűrűket metszettünk a főhörgőkből. Az így kilakított gyűrűket függőlegesen 2 kis L-alakú platinahorogra helyeztük, melyek közül az egyiket a tönkhöz stabilan rögzítettük, míg a másikat az izometrikus tenziókat regisztráló transducerrel kötöttük össze. A kísérletet termosztáttal állandó hőmérsékleten (37 ± 0.2ºC-os) tartott Krebs oldatban (összetétele mM-ban: NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5, glükóz 11) végeztük el (5 ml) TSZ 02, EXPERIMETRIA UK, London készülékkel. A szervfürdőt 95% O_2-t és 5% CO_2-t tartalmazó gázeleggyel (Carbogen) oxigenizáltuk, és 7.40 ± 0.05-os pH-n tartottuk. A szegmensek kontraktilis aktivitását téringerléssel (20 V, 100 stimulus, 0.1 ms, 20 Hz, és 15 mN előfeszítés) tanulmányoztuk. Vizsgáltuk az alkalmazott téringerlési protokoll szelektivitását. Némely bronchusgyűrűt 10 percen keresztül tetrodotoxinnal (TTX) előkezeltünk. A TTX blokkolja a gyors Na^+-csatornákat. 1 μM TTX jelenlétében a fenti téringerlési paraméterek alkalmazásakor nem kaptunk választ.

5.5 A neuropeptid receptorok traszkripciós mintázatának vizsgálata

5.5.1 *RNS izolálás*

Patkány tüdőt homogenizáltunk majd totál RNSt izoláltunk a gyártó (RNeasy Mini Kit Quiagene Inc.) utasításának megfelelően. Háromszáz mg szövethez 600 μl, 10%-os merkapto-etanolt tartalmazó RLT puffert adtunk. A szövetet homogenizáltuk, majd 2x3 percen keresztül centrifugáltuk (13000 rpm). A felülúszót új csőbe tettük, majd azonos térfogatú 70%-os alkoholt tettünk hozzá. Centrifugálás nélkül pipettával összekevertük, majd a minta 700 μl-ét gyűjtőcsőbe tettük. Két részletben centrifugáltuk le a mintát, (10000 rpm, 15 sec). A gyűjtőcső oszlopán átfolyó oldatot eldobjuk. A gyűjtőcsőbe 700 μl RW1 puffert tettünk, majd 10000 rpm-en 15 sec-ig centrifugáltuk a mintát. A gyűjtőcső oszlopán átfolyó oldatot és a gyűjtőcsövet eldobtuk. A mintát új gyűjtőcsőbe helyeztük, majd 500 μl RPE puffert adtunk hozzá majd centrifugáltuk (10000 rpm, 15 sec). A gyűjtőcső oszlopán átfolyó oldatot eldobtuk. Újabb 500 μl RPE puffert adtunk a mintához, majd 2 percen keresztül centrifugáltuk 10000 rpm-el. A gyűjtőcső oszlopán átfolyó oldatot és a gyűjtőcsövet eldobtuk.

Harminc mikroliter RNáz-mentes vízzel átcentrifugáltuk a mintát (1 perc, 13000 rpm), majd 50 μl RNáz-mentes vízzel centrifugáltuk (1 perc, 13000 rpm). Az izolált totál RNS-t

1%-os agaróz gélen futtattuk, valamint megmértük a koncentrációját (Nanodrop-1000). A quantitatív mérésekhez 200 μg RNS-t használtunk fel. A tiszta RNS minták OD260/OD280 arányai nagyobbak voltak, mint 1.9.

5.5.2 *Primerek*

A vizsgált receptorokhoz primereket a primer3 internetes oldal segítségével terveztük (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (2006. április 19.-én elérhető). A vizsgált gének a következők voltak: nk1, nk2, cgrpr, sstr4. Belső standardnak a béta-2-mikroglobulint b2m-t használtuk.

A felhasznált primerek szekvenciái a következőek voltak:

nk1 receptor forward: tgggcaacgtagtggtgata, reverse: cacggctgtcatggagtaga;
nk2 receptor forward:ggagagtcaaccggtgtcat, reverse: ccgagcaccattctgttttt;
cgrpr receptor forward: agaacttgaacgccatcacc, reverse: ggatctcaacagcggtcatt;
sstr4 forward gccactgtcaaccatgtgtc, reverse: tcttcctcagcacctccagt;
b2m forward: acttcctcaactgctacg, reverse: tggtgtgctcattgctat

5.6 Quantitative real-time polymerase chain reaction (QRT-PCR)

A QRT-PCR módszer elméleti alpjai jól ismertek. Röviden: reverz transzkripció során a DNS amplifikálódik. Az amplifikáció során a SyberGreenI festék a DNS kis árkába reverzibilisen kötődik. A festék UV fénnyel gerjeszthető. A detektor minden ciklus után vizsgálja a fluoreszcencia növekedését. A beállított küszöb eléréséhez szükséges ciklusszámot (Ct) a számítógép kijelzi. A LightCycler beállításait a gyártó által előírt módon végeztük el. Az RNS minták vizsgálatát a gyártó által forgalomba hozott kit segítségével, a gyártó által ajánlott módon használtuk fel. A LightCycler beállításai a következők voltak: reverz transzkripció: 61ºC, 1200 s; denaturáció 95ºC, 30 s; amplifikáció: 1: 95 ºC 1 s, 2: 56 ºC 5 s, 3: 72ºC 13 s; ciklus szám:45; olvadási görbe analízise: 1: 95 ºC 5 s, 2: 65 ºC 15 s, 3: 95ºC 0 s; hűtés: 40 ºC, 30 s.

Csak azokat az eredményeket fogadtuk el, amelyben az olvadási görbe analízisekor határozott csúcsot, valamint a gélelektroforézis során jól látható produktumot láttunk. A

cisplatin kezelés a vizsgált gének transzkripciós mintázatára gyakorolt hatását relatív analízissel vizsgáltuk

5.6.1 *Relatív analízis ($2^{-\Delta\Delta C}{}_t$)*

Cisplatinnal kezelt, valamint a kontroll csoportokból származó mintákból izolált mRNS segítségével A $2^{-\Delta\Delta C}{}_t$ módszer segítségével vizsgáltuk a cisplatinnak a vizsgált gének expressziójára gyakorolt hatását. A kezelés után kapott transzkripciós mintázatot a kontroll állatokban megfigyelt transzkripciós mintázathoz hasonlítottuk.

Első lépésben a kontroll csoport mintáit vizsgáltuk. A receptorok Ct értékeiből kivontuk a b2m Ct értékeit.

1: $\Delta Ct_{kontroll}=Ct_{receptorkontroll}-Ct_{b2mkontroll}$

Második lépésben a kezelt állatok csoportjait vizsgáltuk.

2: $\Delta Ct_{vizsgált}=Ct_{receptorvizsgált}-Ct_{b2mvizsgált}$

Harmadik lépésben kivontuk a kezelt állatok ΔCt értkeiből a kontroll értékeit.

3: $\Delta\Delta Ct=Ct_{vizsgált}-Ct_{kontroll}$

A számításokat mindkét kezelt csoport esetén elvégeztük. A ΔΔCt értékek változása az expresszió mértékét jelzi. Pozitív ΔΔCt értéknél downregulációt tapasztalunk, negatív ΔΔCt értéknél pedig upregulációt (Radonic és mtsai 2004). A kapott értékeket a $2^{-\Delta\Delta C}{}_t$ módszerrel normalizáljuk (Bernard és Wittwer 2002). Minden csoportból négy állatot vizsgáltunk meg, az ezekből származó adatok egymás ismétlésének feleltek meg.

5.7 Radioimmunoassay (RIA) tanulmányok

5.7.1 *Tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek vizsgálata*

A Wistar patkányok kivéreztetése után a tracheák alsó harmadát a főbronchusokkal együtt eltávolítottuk, megtisztítottuk a környező kötőszövetektől és a zsírszövettől, majd 60

percen keresztül oxigenizált Krebs oldatban perfundáltuk (37 ºC, pH = 7,4 ± 0,05). Mértük a téringerlés (40 V, 0,1 ms, 10 Hz, 120 s, 1200 stimulus) hatására felszabaduló szenzoros neuropeptidek koncentrációjának változását szervfürdőben. A CGRP, SP, SOM koncentrációját 200 µl szervfürdőből határoztuk meg RIA módszerrel. Téringerlés után 2 perccel történt a mintavétel. A módszert Dr. Németh József fejlesztette ki a Pécsi Tudományegyetemen (Nemeth és mtsai 1996).

5.8 A plazma neuropeptid szint meghatározása

A plazma CGRP, SP, SOM szint meghatározása RIA segítségével történt. A módszert laboratóriumunkban fejlesztettük ki (Nemeth és mtsai 1999b). Röviden: artériás vért vettünk (3ml/állat), majd EDTA-t (6 mg) és Trasylolt (1 IU) tartalmazó, jégen tartott csőbe tettük. A mintákat 4 ºC-on (2000 rpm, 10 perc) centrifugáltuk. 1 ml plazma SOM mennyiségét 3 ml abszolút etanollal extraháltuk. Precipitáció után ugyanezen paraméterek mellett centriugáltuk másodjára is a mintát. A felülúszót leszívtuk, majd a minta maradékát nitrogén alatt szárítottuk (Nemeth és mtsai 1998).

5.9 Perifériális inzulin rezisztencia (HOMA-IR) és β-sejt funkció vizsgálata (HOMA-%B)

Az éhgyomri vércukorszintet (FPG) Accu-Chek segítségével, glükóz oxidáz módszerrel végeztük, míg a plazma inzulin szinteket (FPI) RIA módszerrel határoztuk meg. A β-sejtek funkcióvizsgálata valamint az inzulin rezisztencia változását a homeosztázis modell (HOMA) kiértékelésével vizsgáltuk. Az FPG (mmol/l) és inzulin koncentrációk (FPI) (µU/ml) ismertében a következő képletek segítségével számítottuk ki a perifériális inzulin rezisztencia (HOMA-IR) és a β-sejt funkció változását (HOMA-%B)(Matthews és mtsai 1985).

HOMA-IR = (FPI X FPG)/22.5
HOMA-%B = (20 X FPI) / (FPG-3.5)

5.10 Statisztika

A bronchomotilitás vizsgálatokból származó eredmények átlagait a szórással (± S.D.) fejeztük ki, melyeket Bonferonni módszere alapján (Wallenstein és mtsai 1980) kidolgozott ismételt méréseket követő módosított t- teszttel ANOVA után vizsgáltunk. A tracheából téringerlés hatására felszabadult neuropeptid mennyiségek változásait párosítatlan Student-t-teszttel vizsgáltuk, az adatok átlagait ± S.D. -vel fejeztük ki, a plazma neuropeptid szintek mennyiségének változásait módosított ANOVA-val majd Student-t teszttel vizsgáltuk. Az adatok normalizációjából fakadó hibákat Man-Whitney U-teszt segítségével küszöböltük ki. Az adatok átlagait a szórás hibájával (± S.E.M.) fejeztük ki. A kapott eredmények közül azokat tekintettük szignifikánsnak, ahol a $p \leq 0{,}05$. A QRT-PCR vizsgálatok átlagait ± S.E.M–el fejeztük ki. Szignifikánsnak tekintettük az expresszió növekedését, ha az expresszió mértéke kétszerese volt a kontrollnak.

6 VIZSGÁLATOK

6.1 Cisplatin kezelés bronchomotilitásra gyakorolt hatása

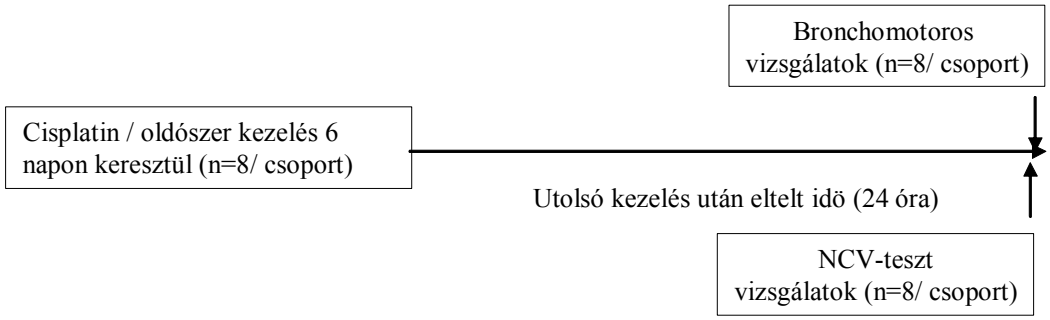
6.1.1 *Kísérleti csoportok*

Cisplatin kezelés bronchomotilitásra gyakorolt hatását 16 hím tengerimalacon vizsgáltuk, amelyeknek 350-400 g volt a súlyuk. Állatházban tartottuk őket (12 óra nappali és 12 óra éjszakai megvilágításában, 22-25 ºC-os hőmérsékleten, 50-70%-os páratartalomban), ketrecenkét két állat volt, standard laboratóriumi takarmányt és csapvizet kaptak. Az állatokat véletlenszerűen két csoportra osztottuk. Az első csoportnak (kontroll) a cisplatin oldószerét (1 ml izotóniás NaCl-t) és 75 mg/kg mannitolt adtunk i.p. naponta egyszer, 6 napon keresztül. A második csoportnak (kezelt csoportnak) 3 mg/kg cisplatint adtunk 75mg/kg mannitollal i.p. naponta egyszer, 6 napon keresztül.

6.1.2 *Kísérleti protokoll*

Mindkét csoportból 8 állatot a kezelés után 24 órával tiopentallal (Trapanal) (50 mg/kg i.p.) elaltattunk. Miután meghatároztuk az állatok n. saphenusán az ingervezetési sebességet, eltávolítottuk a tracheákat a főbronchusokkal. Az izometriás tenzióváltozásokat 2 mm vastagságú bronchus szegmenseken tanulmányoztuk. A mintákat Krebs-oldatban 60 percig equilibrálódni hagytuk, majd ezt követően téringereléssel (100 stimulus, 20V, 0.1 ms 20Hz) bronchokonstikciót indukáltunk. A gyűrűket ezek után 4 μM guanethidinnel és 1 μM atropinnal (non-adrenerg non-kolinerg [NANC] oldat) 60 percig inkubáltuk, majd a téringerlő protokollt megismételtük. Ezek után a preparátumokat nem szelektív nitrogén-monoxid-szintáz (NOS) gátlóval (30 μM L-NAME) kezeltük (Rees és mtsai 1990), majd a téringerlési protokollt megismételtük. A beavatkozások után addig mostuk a preparátumokat, míg a TI kezdeti válaszok újra meg nem jelentek. A capsaicin és a szuper-oxid-dizmutáz (SOD) hatását más-más preparátumokon tanulmányoztuk. Vizsgáltuk, befolyásolják-e a szenzoros rostok a TI által kiváltott kontraktilis válaszokat. A kontroll állatokból származó gyűrűket TI bronchomotilitás válaszok kialakulása után 30 percnél hosszabb ideig tartó 100 μM-os capsaicin kezelésnek tettük ki. Ezt 30 perces kimosási periódus követte és a stimulációs protokollt megismételtük. A szuperoxid anionok TI NANC kontraktilis válaszokra gyakorolt hatását SOD (40 U/ml) hiányában vagy jelenlétében tanulmányoztuk. A kísérleteket kétszer

ismételtük, így reprodukáltuk a TI bronchomotilitás változásokat. Az alkalmazott téringerlési protokoll szelektivitását tetradotoxin (TTX) előkezeléssel bizonyítottuk. (10 percig tartó TTX előkezeklés után nem alakult ki TI bronchokonstrikció).



3. ábra Az alkalmazott kísérleti protokoll sematikus ábrázolása. Az állatokat véletlenszerűen két csoportba osztottuk. A kezelt csoport 6 napon keresztül a 75 mg/kg mannitolon kívül 3 mg/kg cisplatint kapott, a kontroll pedig a cisplatin oldószerét. A kezelés után 1 nappal elvégeztük a vezetési sebesség mérés (nerve conduction velocity, NCV) és a bronchomotilitás vizsgálatokat.

7 Eredmények

7.1 Kizárás

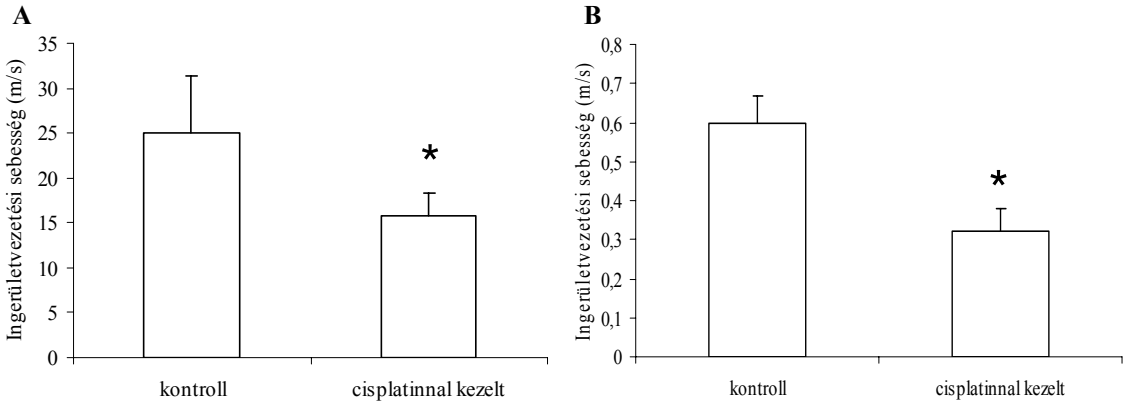
A cisplatinnal kezelt csoport 4 állatát ki kellett zárni a kísérletből. Kettő állatot azért, mert cisplatin kezelés hatására sem az „A", sem a „C" rostokon sem csökkent az NCV. A harmadikat pedig azért, mert a kezelés hatására kialakuló tüdőkárosodás (feltételezhetően pneumónia) légzési elégtelenséghez vezetett. A negyedik állatban kiterjedt bőrléziók alakultak ki.

7.2 A testtömeg és rektális hőmérséklet

A cisplatinnal kezelt állatok testtömege a 381 ± 41g -ról 308 ± 31g -ra csökkent ($p \leq 0.05$). A kontroll csoport testtömege a kezelés alatt nem változott szignifikánsan (394 ± 25g -ról 415 ± 31g –ra). A rektális hőmérséklet egyik csoportban sem változott.

7.3 Az ingerületvezetési sebesség mérésének eredményei

A 4. ábra mutatja a cisplatin- indukálta idegen mért NCV csökkenését. A bal oldalon látható az „A" rostokon, és jobb oldalon a „C" rostokon mért NCV. Küszöb feletti stimuláció „A" rostokon 0.5 V, 5 Hz „C" rostokon 3 V, 5 Hz volt.

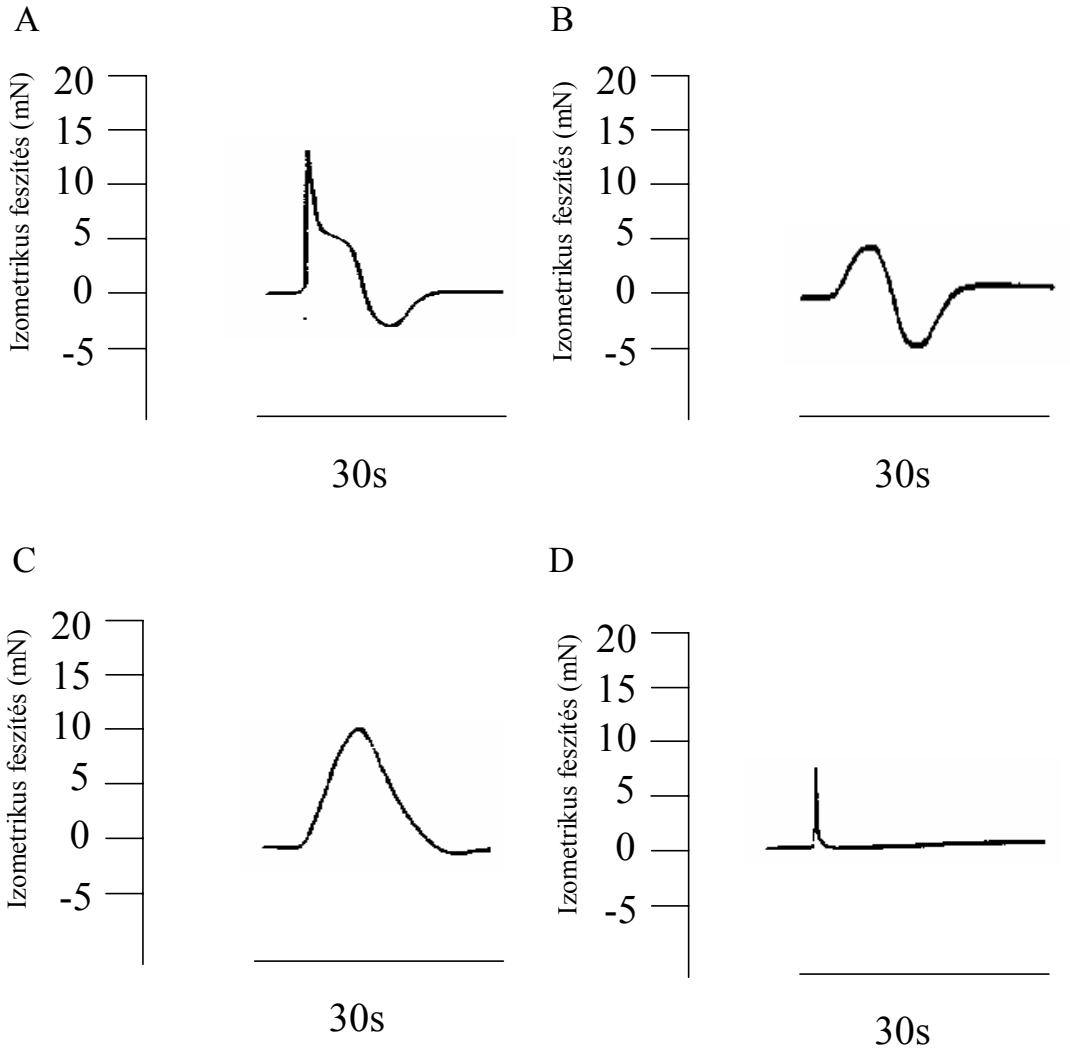


4. ábra N. saphenuson mért NCV. A cisplatin- indukálta NCV csökkenés a n. saphenus gyors „A" (bal panel), és lassú vezetésű „C" (jobb panel) rostjain. A méréseket 24 órával a 6. intraperitoneális cisplatin injekció (3 mg/kg/nap) adása után végeztük el. Az adatokat átlaggal és szórással (± S.D.) fejeztük ki, egy csoportban 8 állat volt. *: a kontrolltól a kezelt szignifikánsan különbözött, $p \leq 0,05$.

7.4 Téringerlés hatására kialakult izometriás tenzióváltozások

7.4.1 *TI bronchomotilitás változások a kontroll csoportban*

A kontroll csoportból származó bronchusgyűrűk Krebs-oldatban téringerlés hatására kétfázisú választ adtak (5. ábra). Az első, kontraktilis válasz magában foglalt egy kezdeti gyors, majd egy lassú választ. A kétfázisú kontrakciót egy lassú relaxációs válasz követte. A gyors kontrakciós komponens 4μM guanetidint 1 μM atropint tartalmazó Krebs-oldatban a (NANC-oldatban) eltűnt. A NANC relaxáció 30 perces 30 μM L-NAME inkubációval gátolható. 100 μM-os, 30 percig tartó capsaicin hatására viszont eltűntek a lassú válaszok, csak a tüskeszerű, gyors kontrakciós fázis maradt.

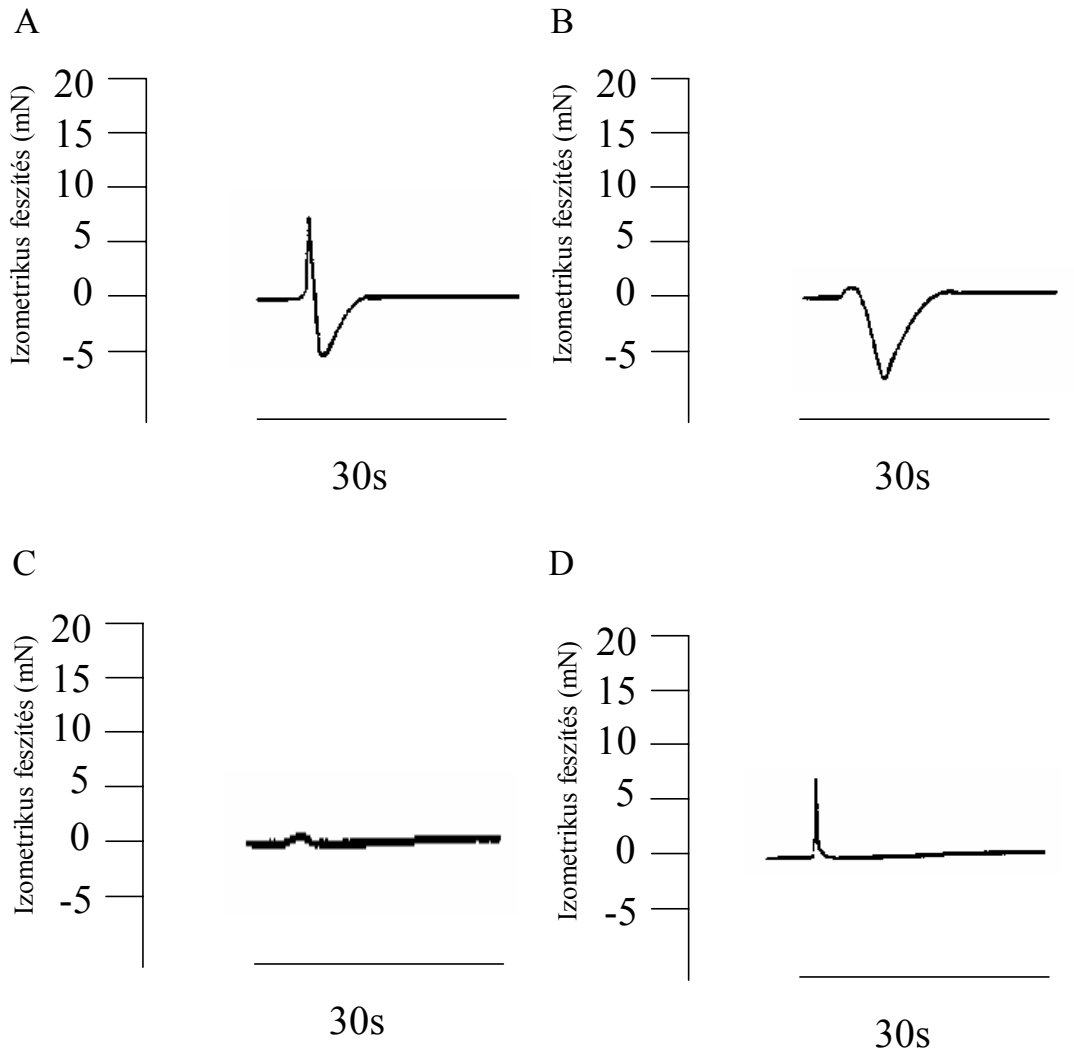


5. ábra TI bronchomotilitás változások. Kontroll csoport. „A" panel: a preparátumok Krebs-oldatban, „B" panel: a preparátumok Krebs-oldatban és NANC-oldatban. „C" panel: NANC-oldat és 30 μM L-NAME, „D" panel: 100 μM capsaicin előkezelés. A téringerlés paraméterei a következők voltak: 20V, 100 stimulus, 0.1 ms, 20 Hz. Az előfeszítés 15 mN volt.

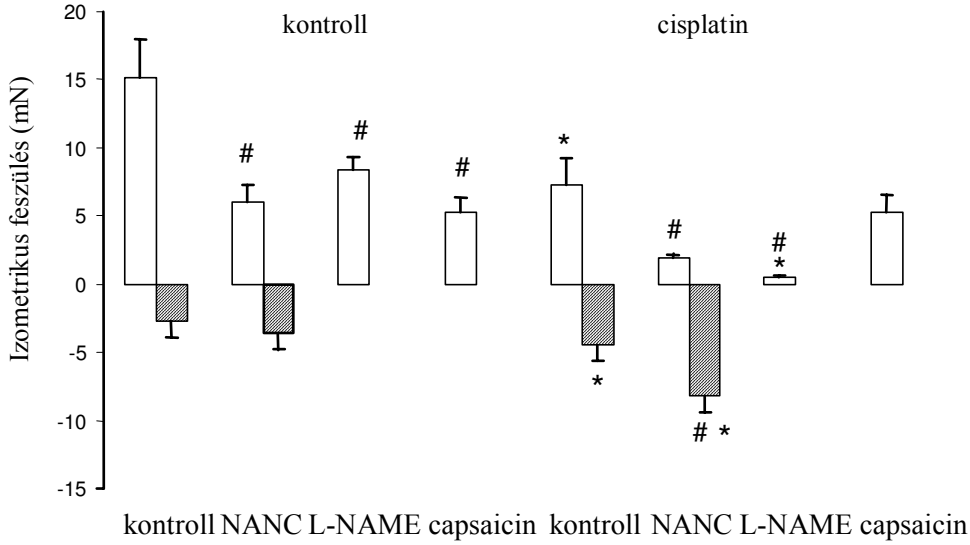
7.4.2 *TI bronchomotilitás változások a cisplatinnal kezelt állatokban*

Cisplatinnal kezelt állatok bronchusgyűrűi téringerlés hatására szintén kétfázisú választ adtak. A kezdeti kontraktilis fázis amplitúdója és hossza is szignifikánsan gyengült a kontrollhoz képest (6. ábra). Fokozódott viszont a relaxáció amplitúdója és hossza. A relaxáció amplitúdójának L-NAME-val szembeni érzékenysége szignifikánsan

megnövekedett. A capsaicin hatására kialakuló kontrakció azonban a cisplatinnal kezelt csoport esetén is tüske alakú maradt.



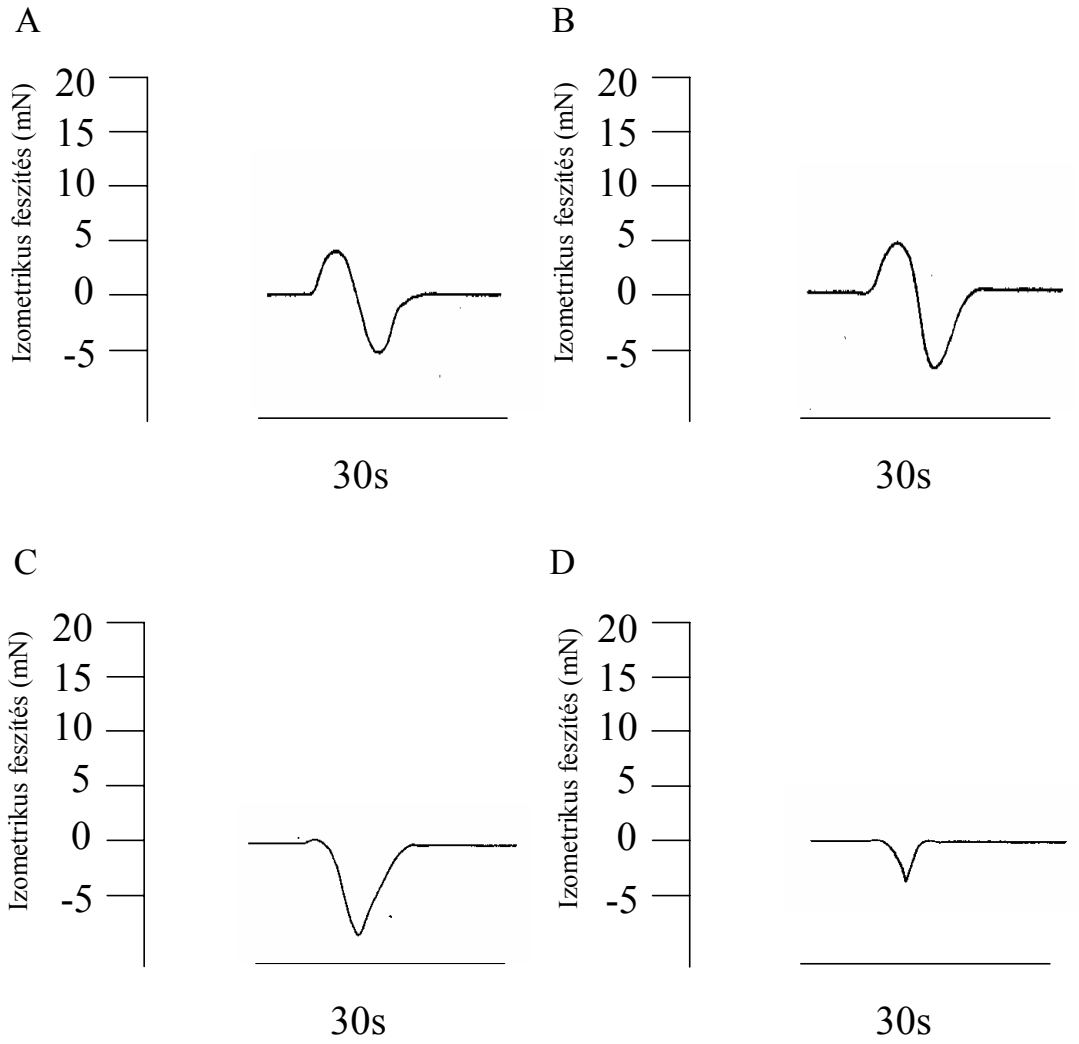
6. ábra TI bronchomotiliás változások. Cisplatinnal kezelt csoport. „A" panel: a preparátumok Krebs-oldatban, „B" panel: a preparátumok Krebs-oldatban és 1 μM guanethidint és 4 μM atropint tartalmazó oldatban (NANC-oldat). „C" panel: NANC oldat és 30 μM L-NAME, „D" panel: 100 μM capsaicin előkezelés. A téringerlés paraméterei a következők voltak: 20V, 100 stimulus, 0.1 ms, 20 Hz. Az előfeszítés 15 mN volt.



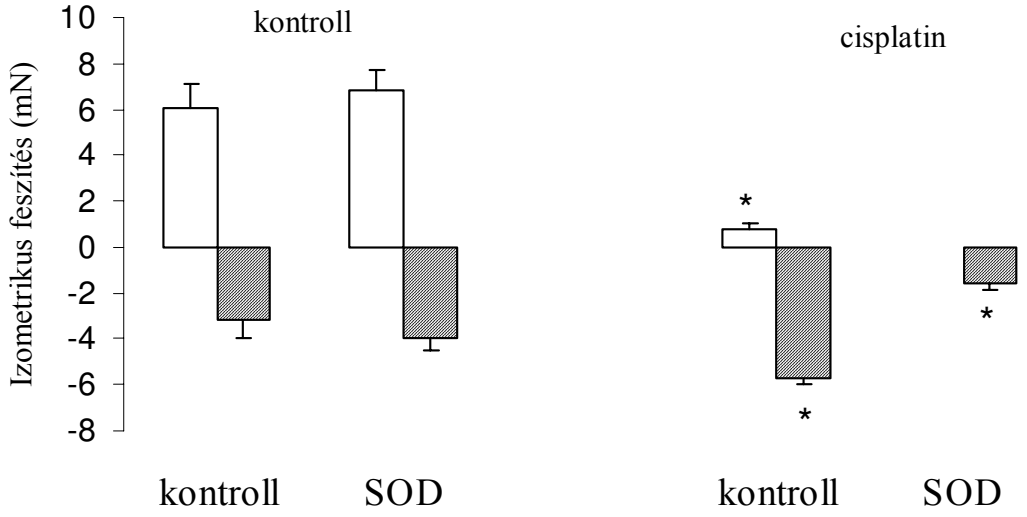
7. ábra TI bronchomotoilitás-változás adatainak átlagainak összefoglalása. Az adatok átlagait ± S.D.-val fejeztük ki. *: TI bronchorelaxáció szignifikánsan különbözik a kontrolltól. #: TI bronchokonstrikció szignifikánsan különbözik a kontrolltól. A téringerlés paraméterei: 20 V, 100 stimulus, 0.1 ms, 20 Hz voltak. Az előfeszítés 15 mN volt. *,#: $p \leq 0.05$.

7.4.3 *A SOD bronchomotilitásra gyakorolt hatása*

A kontroll csoportból származó bronchuspreparátumok TI NANC kontraktilis válaszait a SOD nem befolyásolta. A cisplatinnal kezelt csoportok preparátumainak relaxációját azonban a SOD jelenléte szignifikánsan gyengítette (8. és 9. ábra).



8. ábra: SOD hatása a TI bronchomotilitásra. „A”-panel: kontroll csoport, NANC-oladt, „B”-panel: kontroll csoport, NANC+ 30 μM L-NAME. „C”-panel: cisplatinnal-kezelt csoport, NANC-oladt, „D”-panel: cisplatinnal-kezelt csoport, NANC+ 30 μM L-NAME. A téringerlés paraméterei a következők voltak: 20 V, 100 stimulus, 0.1 ms, 20 Hz. Az előfeszítés 15 mN volt.



9. ábra SOD hatása a TI bronchomotilitásra. Az adatok átlagait ± S.D.-val fejeztük ki. A téringerlés paraméterei a következők voltak: 20 V, 100 stimulus, 0.1 ms, 20 Hz. Az előfeszítés 15 mN volt. *: a kontroll csoporttól a cisplatinnal kezelt csoport szignifikánsan különbözött, $p \leq 0{,}05$.

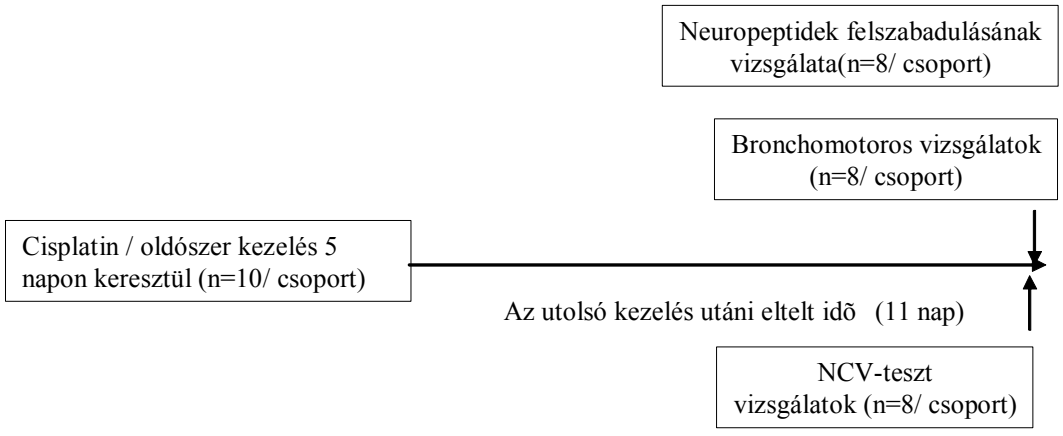
8 Cisplatin kezelés hatása a tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek mennyiségére

8.1 Kísérleti csoportok

A kísérlet során 20 hím, 270-320 g súlyú Wistar patkányt használtunk fel. Állatházban tartottuk őket (12 óra nappali és 12 óra éjszakai megvilágításban, 22-25 ºC-os hőmérsékleten, 50-70 %-os páratartalomban). Két állat volt egy ketrecben, általános laboratóriumi takarmányt és csapvizet kaptak. Az állatokat véletlenszerűen két csoportra osztottuk. A kontroll csoportnak a cisplatin oldószerét (1ml izotónikus NaCl-t) és 75 mg/kg mannitolt adtunk i.p. naponta egyszer, 5 napon keresztül. A kezelt csoportnak 1.5 mg/kg cisplatint adtunk 75 mg/kg mannitollal i.p. naponta egyszer, 5 napon keresztül (Bardos és mtsai 2003). A kezelés után a cisplatin hatásának kialakulásáig 11 napot vártunk.

8.2 A kísérleti protokoll

Mindkét csoportból 8 állatot elalttatunk tiopentallal (Trapanal) (50 mg/kg i.p.) a kezelés után 11 nappal. Miután megmértük az állatok n. saphenusának ingerületvezetési sebességét, eltávolítottuk a tracheákat és a főbronchusokat. Az izometriás feszítés változásokat 2 mm vastagságú szegmenseken végeztük. A tracheák többi részét a szenzoros neuropeptidek vizsgálatára használtuk fel.



10. ábra Az alkalmazott kísérleti protokoll sematikusan ábrázolva. Az állatokat véletlenszerűen két csoportba osztottuk. A kezelt csoport 5 napon keresztül a 75 mg/kg mannitolon kívül 1,5 mg/kg cisplatint kapott, a kontroll pedig a cisplatin oldószerét. A kezelés után 11 nappal elvégeztük az NCV, a bronchomotilitás, és a RIA vizsgálatokat.

9 Eredmények

9.1 Állatok kizárása

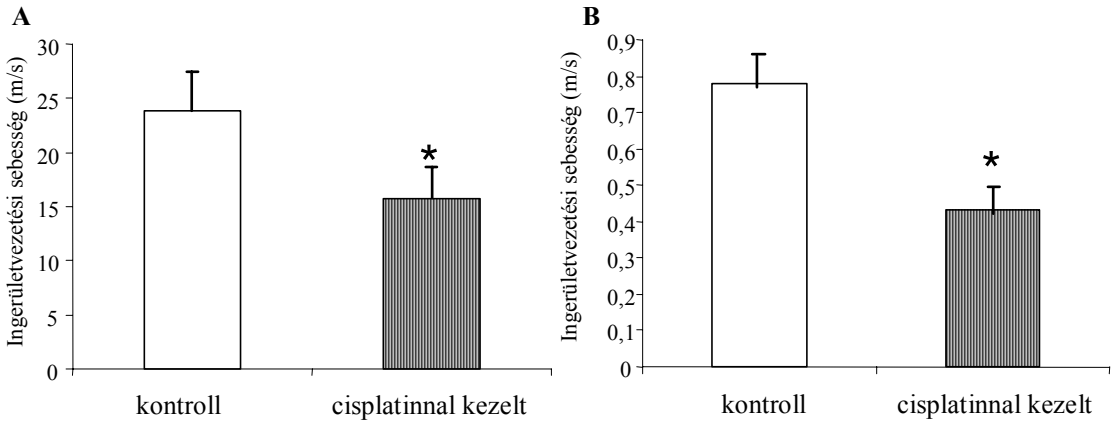
Két cisplatinnal kezelt állatot zártunk ki a kísérletből. Az egyik állatot azért kellett kizárni, mert elpusztult, a másik állatot pedig azért, mert cisplatin kezelés hatására sem az „A", sem a „C" rostokon sem csökkent az NCV.

9.2 Testtömeg és a rektális hőmérséklet mérésének eredménye

A testtömeg a kezdeti 294 ± 22 g –ról 231 ± 19 g-ra csökkent ($p \leq 0.05$) a cisplatinnal kezelt csoportnál. A kontroll állatok testsúlya 289 ± 23 g-ról 306 ± 20 g-ra növekedett. A rektális hőmérséklet egyik csoportnál sem változott.

9.3 Ingerületvezetési sebesség mérésének eredményei

Cisplatin kezelés hatására mind a gyors, „A" rostok, mind a „C" rostok ingerületvezetési sebessége a kontrollhoz képest csökkent (11.ábra).

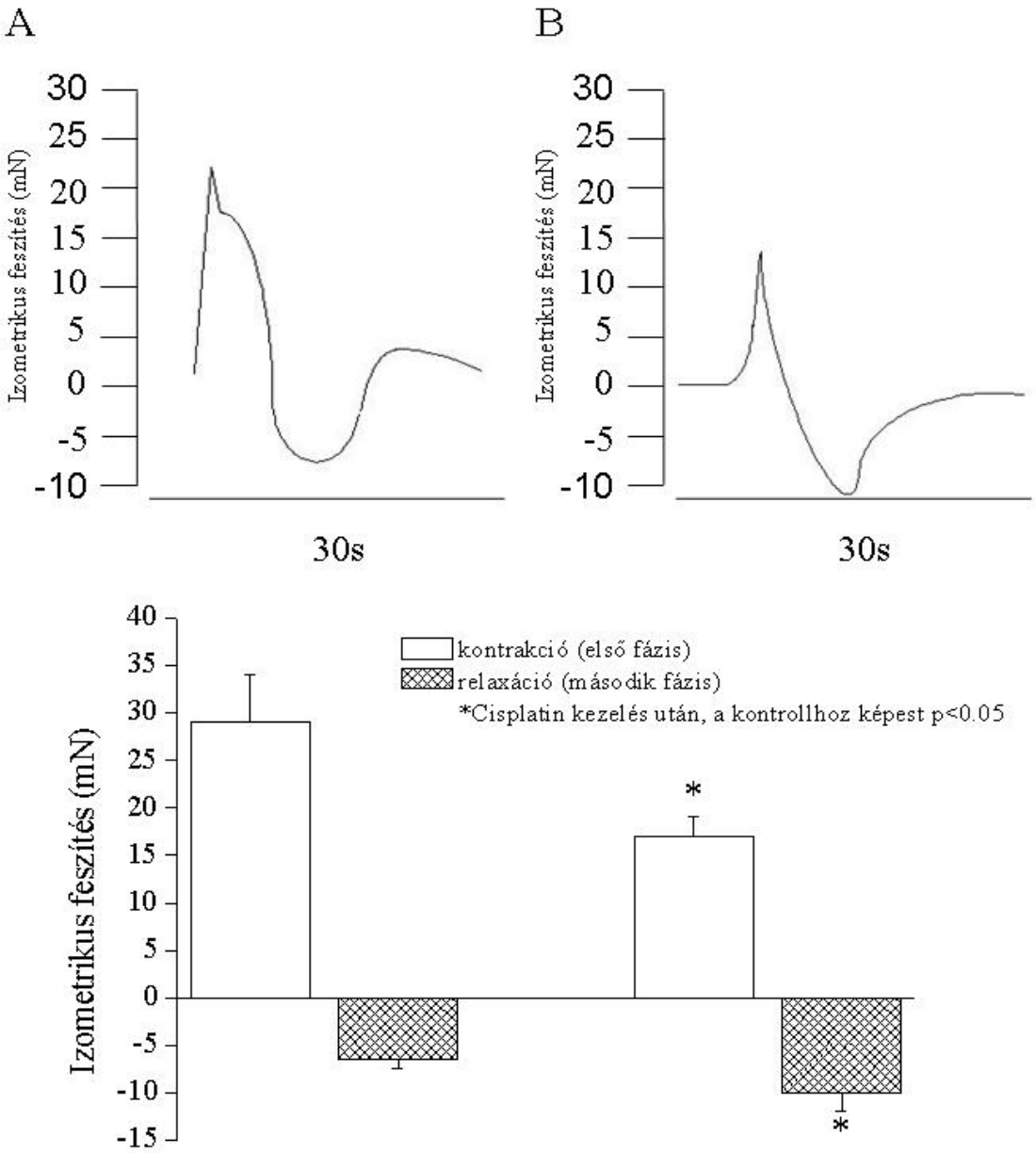


11. ábra Cisplatin kezelés mind a gyors, myelinizált „A" rostok („A" panel), mind a velőshüvely nélküli „C" rostok („B" panel) ingerületvezetési sebességét csökkenti. A vizsgálatokat 11 nappal az utolsó cisplatin injekció ill. fiziológiás sóoldat után végeztük el. Az adatokat ± S.D.-val fejeztük ki, csoportonként 8 állatot használtunk fel. Az ingerlési paraméterek a következőek voltak: A rostoknál küszöb feletti stimuláció „A" rostnál 0.5 V, 5 Hz, „C" rostnál 3 V, 5 Hz volt. *: a kontroll csoporttól a kezelt csoport szignifikánsan különbözött, $p \leq 0.05$.

9.4 Izometriás tenzióváltozások vizsgálata

A kontroll csoportból származó bronchusgyűrűk Krebs-oldatban téringerlés (100 stimulus, 20 V, 0.1 ms, 20 Hz) hatására kétfázisú választ adtak (12. ábra). Az első, kontraktilis válasz magában foglalt egy kezdeti gyors, majd egy lassú választ. A kétfázisú kontrakciót egy lassú relaxációs válasz követett.

A cisplatinnal kezelt állatok bronchusainak TI bronchokonstrikciója a kontrollhoz képest csökkent. Az alkalmazott kezelés mellett a kontrollhoz képest a relaxáció időtartama csökkent, amplitúdója pedig nőtt. TTX előinkubáció után téringerlés hatására nem alakult ki bronchokonstrikció.

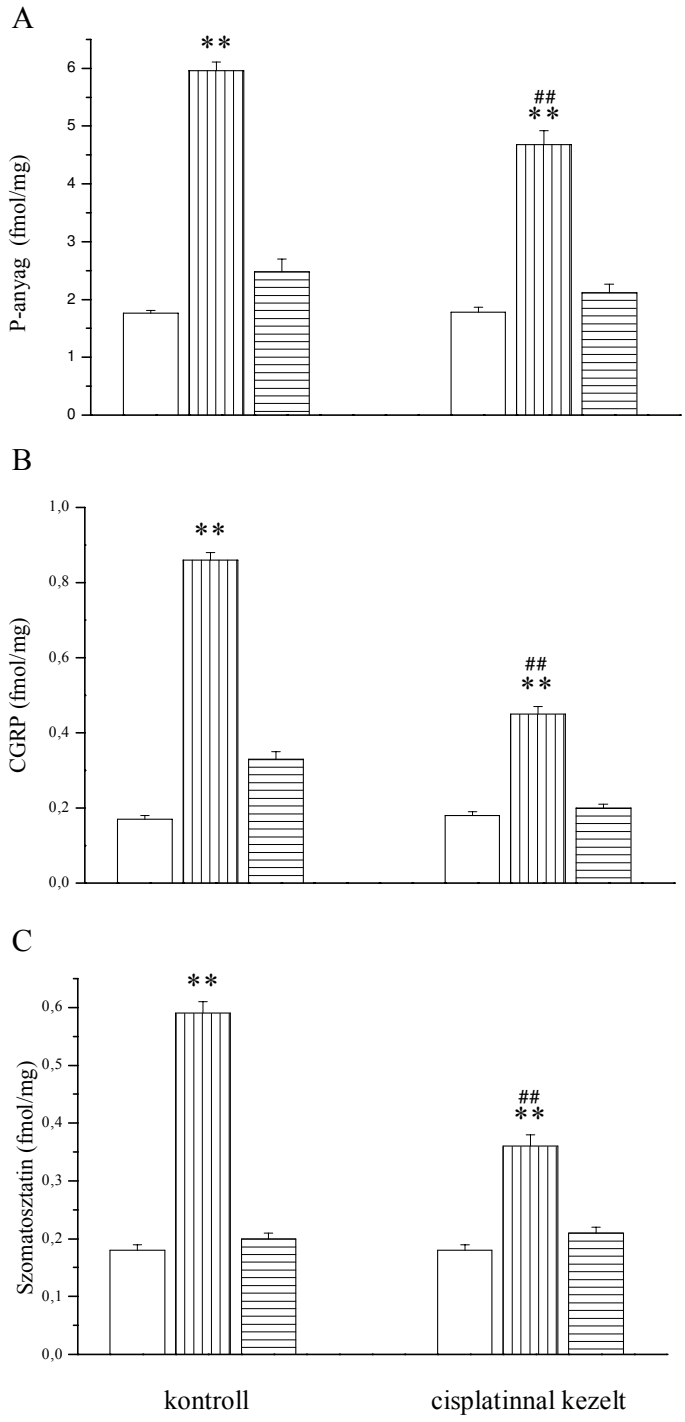


12. ábra Eredeti regisztrátumok (felül) és az adatok összefoglalása (alul). Az ábra jobb oldalán a cisplatinnal kezelt állatok bronchusgyűrűinek téringerlés indukálta bronchomotilitás változása látható, míg a bal oldalon kontroll állatoké. Csoportonként 8 állatot használtunk fel, az adatok átlagát ± S.D.-val fejeztük ki. *: $p \leq 0{,}05$.

9.5 Szenzoros neuropeptidek (CGRP, SOM, SP) vizsgálata

Téringerlés (100 stimulus, 20 V, 0.1 ms, 20 Hz) hatására patkány tracheából felszabaduló SOM, CGRP és SP mennyiségét szervfürdőből RIA-val határoztuk meg. A kontroll csoport tracheáiból téringerlés hatására felszabaduló SOM, CGRP és SP a kezdeti 0.18 ± 0.01; 0.17 ± 0.01 és 0.86 ± 0.02-ről 0.59 ± 0.02; 1.77 ± 0.04 és 5.96 ± 0.03 fmol/mg

nedves tömegre változott. A cisplatinnal kezelt csoport tracheáiból téringerlés hatására felszabaduló SOM, CGRP és SP 0.36 ± 0.02; 0.45 ± 0.02 és 4.68 ± 0.24 fmol/mg nedves tömegre csökkent.



13. ábra: Téringerlés hatására tracheából felszabaduló szenzoros neuropeptidek változására kontroll és cisplatinnal kezelt állatokban. A meghatározások a téringerlés előtt (üres oszlop), közvetlenül a téringerlés után (csíkos oszlop), és két perccel a téringerlés után (keresztbe sávozott oszlop). A téringerlés paraméterei a következők voltak: 20 V, 100 stimulus, 0.1 ms, 20 Hz Az adatok átlagát ± S.D.-val fejeztük ki. A méréseket csoportonként 8 állaton végeztük el. *#: A kontrollhoz képest szignifikáns különbség, $p \leq 0.05$. **## A kontrollhoz képest szignifikáns különbség, $p \leq 0.01$.

10 A cisplatin kezelés hatása a plazma szenzoros neuropeptidek szintjére, vércukorszintre, és neuropeptid receptorok transzkripciós mintázatára

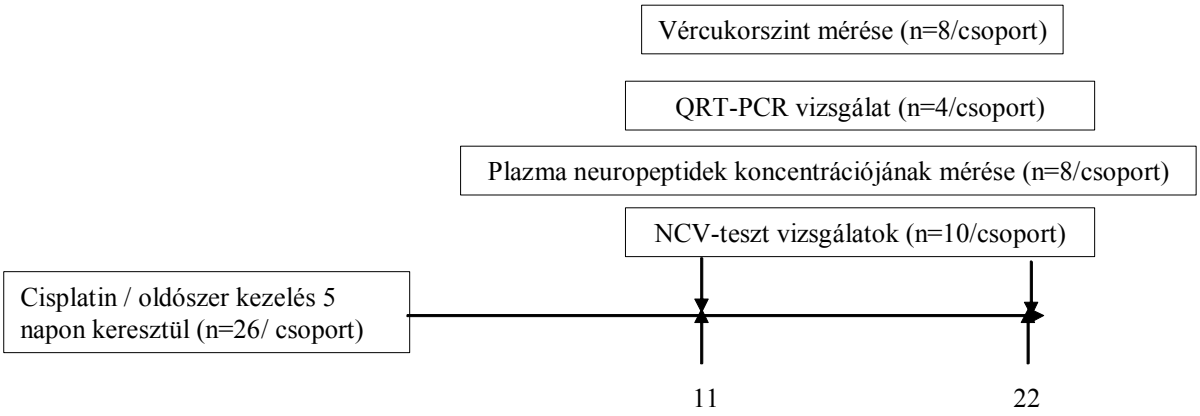
10.1 Kísérleti csoportok

Ötvenkét hím 270-320 g tömegű hím Wistar patkányt használtunk fel a kísérlethez. Állatházban tartottuk őket.(12 óra nappali és 12 óra éjszakai megvilágításban, 22-25 ºC-os hőmérsékleten, 50-70%-os levegőnedvességben), ketrecenként két állat volt, általános laboratóriumi takarmányt, csapvizet tetszés szerinti mennyiségben kaptak. Az állatokat véletlenszerűen két csoportra osztottuk. Az első csoportnak (kontroll) a cisplatin oldószerét (1 ml fiziológiás sóoldatot) és 75 mg/kg mannitolt adtunk i.p. naponta egyszer 5 napon keresztül. A második csoportnak (kezelt csoportnak) 1.5 mg/kg cisplatint adtunk 75 mg/kg mannitollal i.p. naponta egyszer, 5 napon keresztül. Mindkét csoportból 16-16 állatot véletlenszerűen kiválasztottunk, és két csoportba osztottunk a RIA és QRT-PCR vizsgálatokhoz.

A kezelés után mindkét csoportot véletlenszerűen kettéosztottuk. Az állatok felét 11 nappal (Bardos és mtsai 2003), míg a másik felét 22 nappal az utolsó kezelés után dolgoztuk fel.

10.2 Kísérleti protokoll

A kísérlet 16. és 27. napján vizsgáltuk a plazma neuropeptideinek változását. Vérvétel után izoláltuk a tüdőket. A csoportokból kiválasztottunk 4-4 állat mintáját, hogy megvizsgáljuk a neuropeptid receptorok transzkripciós mintázatát. A kezelés után 11 és 22 nappal izoláltunk totál RNS-t. Az ingerületvezetési sebesség méréséhez 36 állatot használtunk fel (14. ábra).

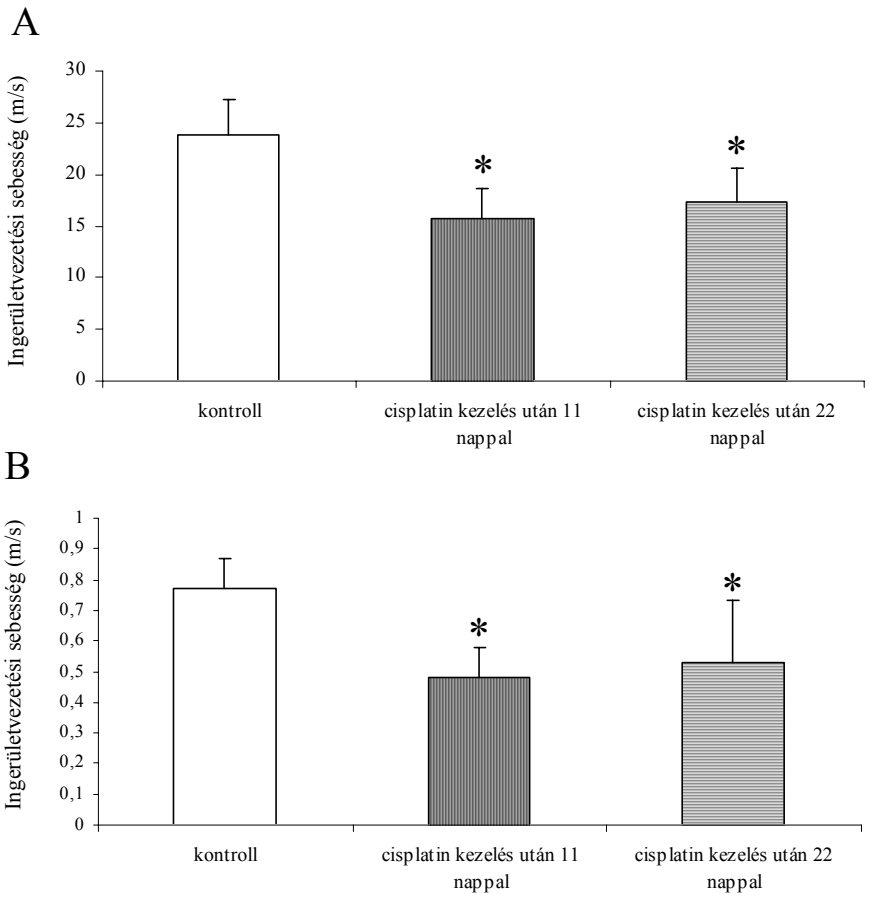


14. ábra A kezelési protokoll sematikus ábrája. Cisplatinnal, ill. annak oldószerével kezeltük az állatokat. QRT-PCR, RIA, NCV, vércukorszint méréseket végeztük a kezelés után 11 és 22 nappal.

11 Eredmények

11.1 Az ingerületvezetési sebesség mérésének eredményei

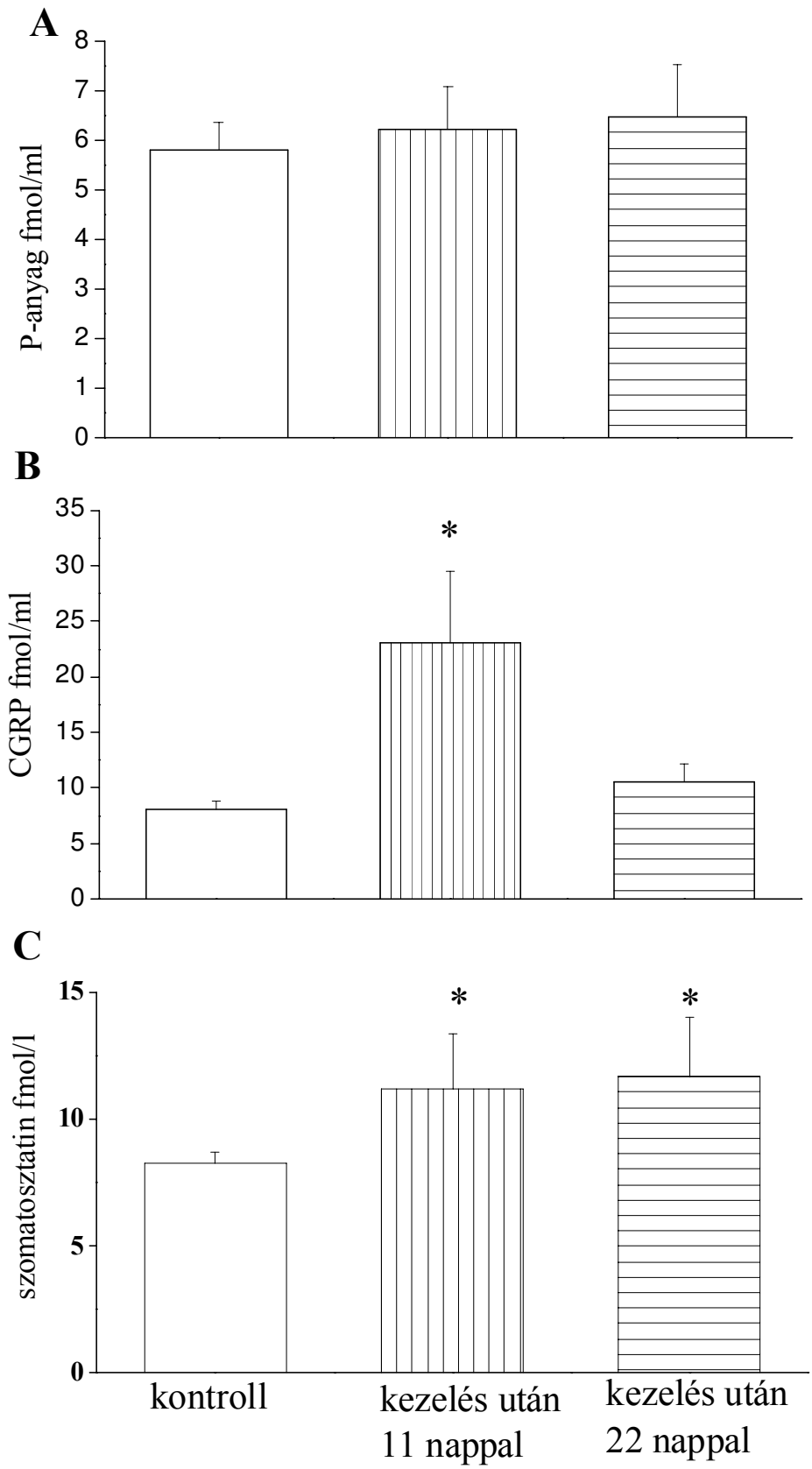
Cisplatin kezelés szignifikánsan csökkentette mind a velőhüvelyes „A" mind a lassú, nem- mielinizált „C" rostok ingerületvezetési sebességét mind a kezelés után 11, mind 22 nap után.



15. ábra Cisplatin kezelés mind a gyors, myelinizált „A" rostok („A" panel), mind a velőhüvely nélküli „C" rostok („B" panel) ingerületvezetési sebességét csökkenti. A vizsgálatokat 11 és 22 nappal az utolsó cisplatin injekció ill. fiziológiás sóoldat után végeztük el. Az adatokat ± S.D.-val fejeztük ki, csoportonként 4 állatot használtunk fel Az ingerlési paraméterek a következők voltak: a rostoknál küszöb feletti stimuláció „A" rostnál 0.5 V, 5 Hz, „C" rostnál 3 V, 5 Hz volt *: a kontroll csoporttól a kezelt csoport szignifikánsan különbözött, $p \leq 0.05$.

11.2 Plazma SOM, CGRP és SP meghatározása

Plazma SOM immunoreaktivitás szignifikánsan növekedett a cisplatin kezelés után mind a 11. mind a 22. napon 8.28 ± 0.44-ról 11.25 ± 2.16 és 11.71 ± 2.33 fmol/ml-ra. A plazma CGRP szint tranziensen növekedett, 8.03 ± 0.79-ről 23.11 ± 6.43 és 10.55 ± 1.58 fmol/ml-ra. Az SP koncentrációja 5.81 ± 0.55-ről 6.23 ± 0.85 és 6.48±1.05 fmol/ml-ra változott.



16. ábra Cisplatin kezelés hatása a plazma SP, (A), CGRP (B), SOM (C) szintre a 16. és a 27. napon. Az adatokat átlag ± S.E.M fejeztük ki. *: A kontrollhoz képest szignifikáns különbség, $p \leq 0.05$.

11.3 Plazma inzulin és glükóz szint meghatározása

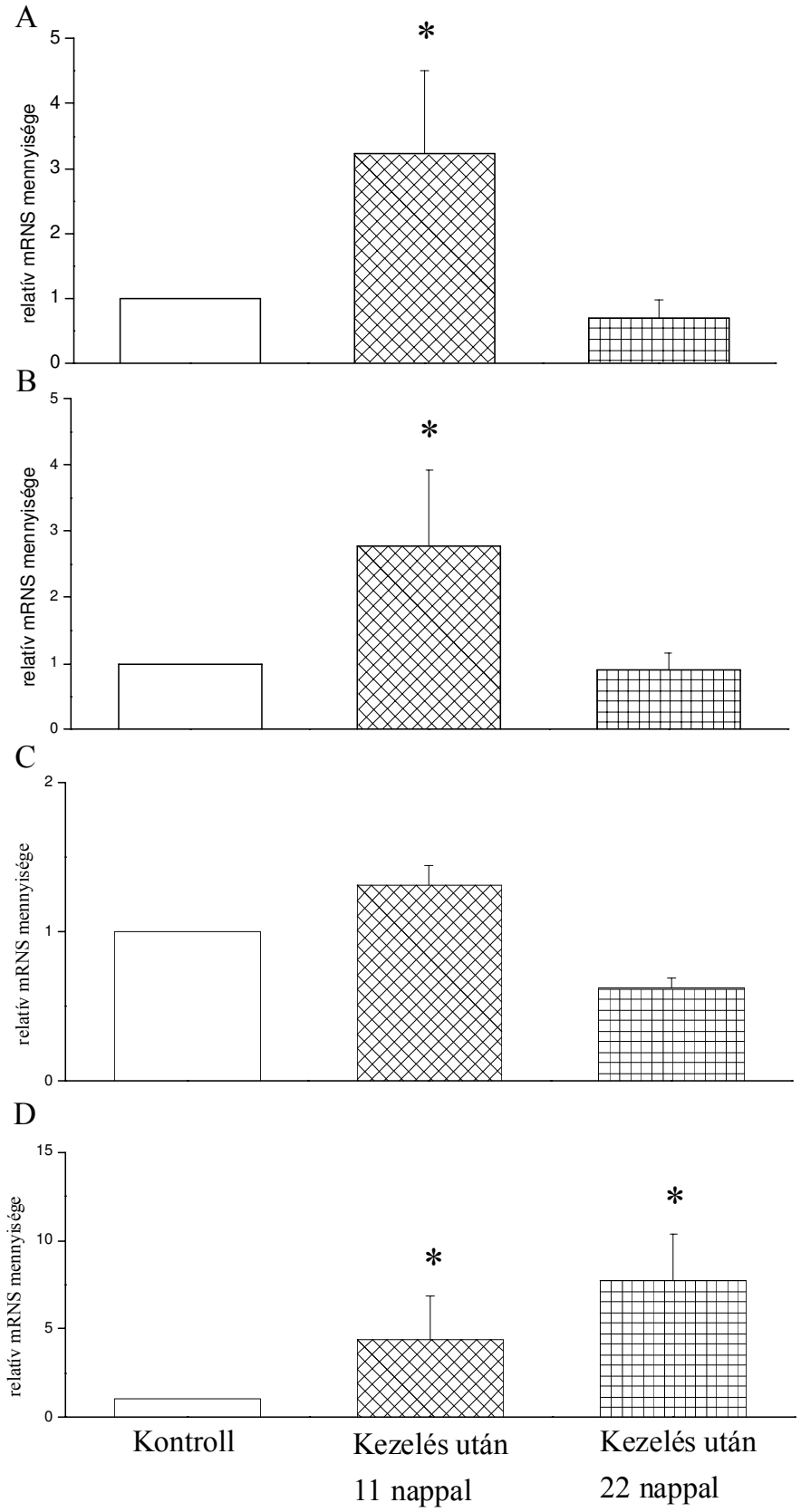
A 4. táblázat mutatja az 5 napos kezelés hatását a plazma inzulin és a glükóz szintre a kontroll és a cisplatinnal kezelt állatokban az utolsó kezelést követő 11. és 22. napon. A kezelés nem befolyásolta sem a plazma inzulin, sem az éhezési vércukorszintet. Ezen eredményekkel párhuzamosan sem a HOMA-IR, sem a HOMA-%B nem változott szignifikánsan.

	Oldószerrel kezelt csoport		Cisplatinnal kezelt csoport	
	16. nap	27. nap	16. nap	27. nap
Plazma inzulin (μU/ml)	9.38 ± 1.36	9.47 ± 0.52	9.63 ± 1.21	9.55 ± 0.42
Plazma glükóz (mmol/l)	4.54 ± 0.15	5.07 ± 0.19	5.68 ± 1.04	6.02 ± 1.00
HOMA-IR	1.89 ± 0.08	2.13±0.03	2.43 ± 0.12	2,55 ± 0.09
HOMA-%B	180.4 ± 19.4	120.6 ± 14.3	88.3 ± 10.1	75.8 ± 6.9

4. táblázat: Plazma inzulin és glükóz szint meghatározása

11.4 QRT-PCR mérések

A kísérlet 16. napján az NK_1, NK_2, CGRP receptorok mRNS-einek expressziója a kontrollhoz képest 3.22 ± 1.29-, 2.78 ± 1.14-, és 1.31 ± 0.14-szeresére növekedett. A kísérlet 27. napján viszont a kontrollhoz képest az NK_1 receptor mRNA 0.69 ± 0.28-ra csökkent, az NK_2 receptor mRNA 0.91 ± 0.24 szeresére, míg a CGRPR mRNA 0.62 ± 0.07-ére. Az SSTR4 mRNA viszont a kontrollhoz képest 4.41 ± 2.48 szorosára növekedett a 16. napon, míg 7.72 ± 2.66 szorosára növekedett a 27. napon.



17. ábra Cisplatin kezelés hatása az nk1 (A), nk2 (B), cgrpr (C), és az sstr4 (D) transzkripciós mintázatára. Minden csoportban 4 állat volt. *: az expresszió legalább kétszeresére nőtt a kontrollhoz képest. Az adatokat ± S.E.M.-ben fejeztük ki.

12 Szenzoros effektor funkciók károsodása diabéteszben

12.1 Kísérleti állatok és csoportok

A kísérlet során 48 hím, 200-210 g-os Wistar patkányt és 12 hím Dunkin-Harley tengerimalacot (400-420 g-t) használtunk fel, állatházban tartottuk őket (12 óra nappali és 12 óra éjszakai megvilágításban, 22- 25 ºC-os hőmérsékleten, 50-70%-os páratartalom). Két állat volt egy ketrecben, általános laboratóriumi takarmányt, csapvizet kaptak tetszés szerinti mennyiségben.

12.1.1 *STZ-kezelés*

Wistar patkányokat véletlenszerűen két csoportra osztottuk. A kontroll csoport állatait az STZ oldószerével kezeltük, míg a kezelteket intravénásan 50 mg/kg streptozotocin (STZ) kezeléssel tettük cukorbeteggé. A 4. hét után az STZ kezelt állatokat két további másik csoportra osztottuk, melyek közül az egyik csoport a kísérlet 4. hetétől kezdve 4 héten keresztül inzulin inplantátumos kezelésben részesült (4 IU/ nap).

12.1.2 *Capsaicin kezelés hatásának vizsgálata*

A szenzoros „C" rostok jelentős részét capszaicin kezeléssel funkcionálisan károsítottuk. Mind a kontroll, mind a diabéteszes állatok közül 6-6 darabot véletlenszerűen kiválasztottunk. Három egymást követő napon 10, 30 és 50 mg/kg capsaicint ill. oldószerét adtuk s.c. naponta egyszer a kezelés 8. hetét követően. A capsaicin oldatot a következő módon készítettük el: 1 rész capsaicinhez attunk 1 rész abszolút alkoholt, majd ehhez adtunk 1 rész Tween 80-t. Az így kapott oldathoz adtunk 8 rész fiziológiás sóoldatot. Az állatokat az utolsó kezelést követő 3 nap után használtuk fel, hogy elkerüljük a capsaicin kezelés aspecifikus szisztémás hatását (Ferdinandy és mtsai 1997, Jancso és mtsai 1967).

12.1.3 *Ovalbumin kezelés hatásának vizsgálata*

12 hím Dunkin-Hurtley tengerimalacot (400-420 g) véletlenszerűen 2 csoportra osztottuk. A kontroll állatok az STZ oldószerét kapták, míg a kezelt állatok 180 mg/kg STZ-t,

(egyszer, i.p.). Az állatokat a kezelés után 4 héttel kétszer, két egymást követő napon i.p. ovalbuminnal (1 ml/kg 5 (%)m/V) aktívan szenzitizáltuk. A 4 hét elteltével az állatokat a tracheakísérletek elvégzéséhez használtuk fel.

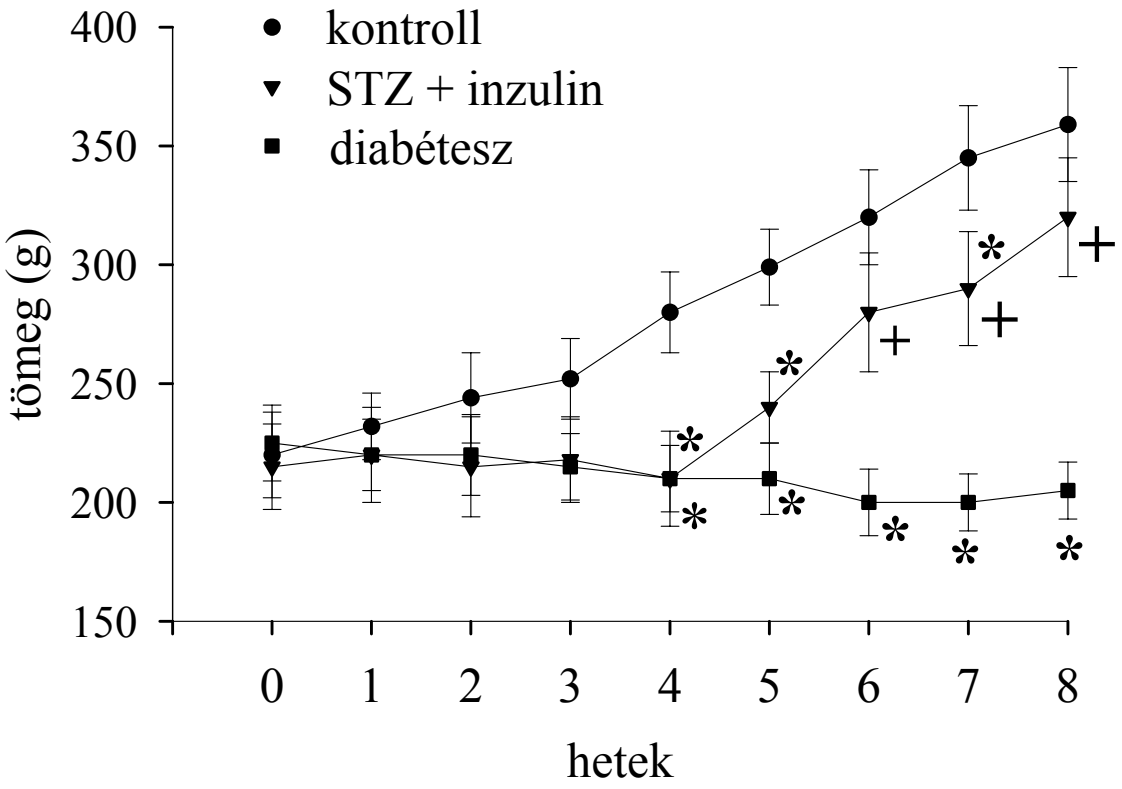
12.2 Kíséleti protokoll

8 héttel az STZ/ oldószer kezelés után, az állatok egy részét kivéreztettük és felhasználtuk in vitro kísérletekhez, laboratóriumi maghatározásokhoz, a többi állaton elvégeztük az NCV vizsgálatokat. 12 órával a vércukor, plazma inzulin és SOM vizsgálatok előtt megvontuk a takarmányt. A plazma inzulin és SOM szintet RIA segítségével határoztuk meg. A tracheák alsó harmadát TI izometriás feszülésváltozások vizsgálatokhoz és TI szenzoros neuropeptidek felszabadadulásának vizsgálatához használtuk fel. NCV méréseket csoportonként 6 állaton végeztük el.

13 Eredmények

13.1 Diabétesz hatása a testtömegre, plazma SOM- és vércukorszintre

A megfigyelt kontroll és inzulin kezelésben részesített cukorbeteg állatok testsúlya fokozatosan növekedett a 8 hetes vizsgálati idő alatt 62 ± 4.1 g-al ill. 58 ± 6.1 g-al. A diabéteszes állatok testsúlya ugyanakkor csökkent 5 ± 2.1 g-al. Azon állatok testsúlya, melyek a 4. héttől inzulin inplantátumot kaptak, a kontrollhoz hasonlóan növekedett (18. ábra).



18. ábra. Az STZ (50 mg/kg iv) kezelés csökkenti a testtömeget. A 4. héttől adott inzulin normalizálja, de nem állítja helyre a testsúly gyarapodását. Csak azoknak az állatoknak a növekedését ábrázoltuk, melyek az STZ, inzulin, oldószeren kívül más kezelést nem kaptak. Az adatok átlagát ± S.D.-val adtuk meg, csoportonként 6 állatot használtunk fel. *: a kontroll csoporttól a kezelt csoport szignifikánsan különbözött, $p \leq 0.05$; +: a diabéteszes csoporttól az inzulin inplantátumot kapott csoport szignifikánsan különbözött, $p \leq 0.05$.

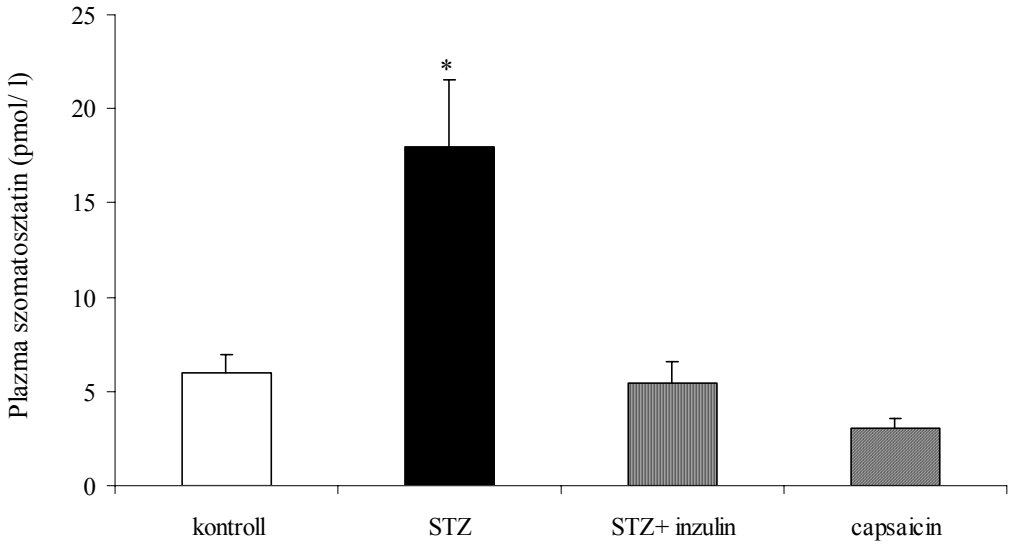
Az STZ oldószerével kezelt tengerimalacoknak megnőtt a testsúlyuk: 45 ± 6.4 g–al a 8 hetes kezelés alatt. Az STZ kezelés után a tengerimalacok a testsúlya nem változott szignifikánsan.

13.2 Éhgyomri vércukor és inzulin szintek

A kísérlet 8. hetén az egészséges kontroll, a diabéteszes és az inzulin-inplantátummal ellátott cukorbeteg állatok éhgyomri vércukorszintjei a következők voltak (mmol/l): 4.4 ± 0.6 17.4 ± 5.5 és 5.0 ± 0.6 ($p \leq 0.001$ cukorbeteg állatok eredményei az egészséges és az inzulin inplantátummal kezelt állatok eredményeihez képest). Az inzulinszintek a következők voltak: 11.4 ± 3.2, 2.0 ± 0.4 és 12.9 ± 3.8 μIU/ml ($p \leq 0.001$ cukorbeteg állatok eredményei az egészséges és az inzulin inplantátummal kezelt állatok eredményeihez képest).

13.3 Plazma SOM szintek

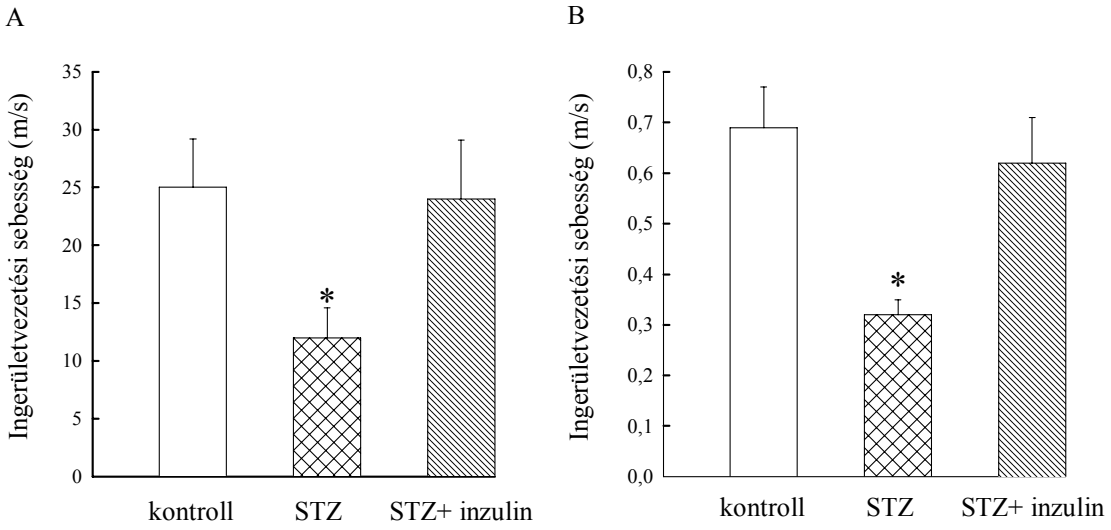
A kontroll patkányokhoz képest az éhgyomri plazma SOM szintje szignifikánsan megnövekedett a cukorbeteg állatokban. A 8. hét végére a 4. héttől adott inzulin inplantáció hatására a plazma SOM szint normalizálódik. A mintavételek a 8. hét végén történtek (19. ábra).



19. ábra STZ kezelés (50mg/kg iv, a mintavétel előtt 8 héttel) és egymást követő capsaicin előkezelés hatása a plazma SOM szintre. A plazma SOM koncentrációját RIA-val határoztuk meg. Az STZ-vel kezelt állatok egy része inzulin inplantátumot kapott a 4. héttől folyamatosan (4 IU/nap). Az adatok átlagát ± S.D-val adtuk meg, csoportonként 6 állatot használtunk fel. *: a kontroll csoporttól a kezelt csoport szignifikánsan különbözött, $p \leq 0.05$.

13.4 Ingerületvezetési sebesség mérés eredményei

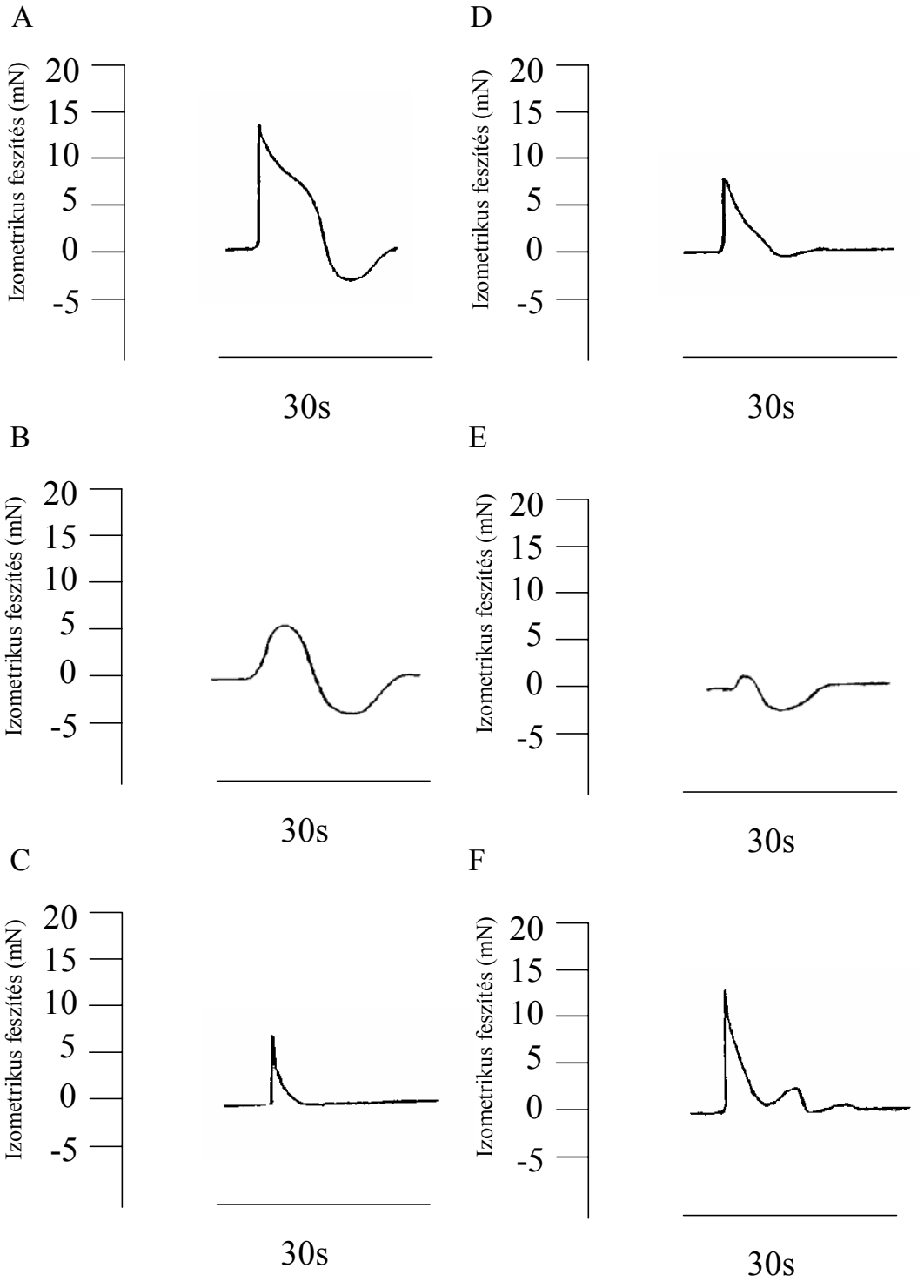
A 20. ábra mutatja a diabétesz hatására kialakult „C" rostok, és „A" rostok ingerületvezetési sebességének változását. A küszöbfeletti impulzus erőssége az „A" rostok esetén 0.5 V, 5 Hz volt, a „C" rostok esetén pedig 3 V, 5 Hz volt. Diabétesz hatására mindkét rost ingerületvezetési sebessége csökkent. Az inzulin implantációs kezelésben részesült állatok n. saphenusán mért NCV normalizálódott, és szignifikánsan nem különbözött a kontroll állatok „A" és „C" rostjain mért NCV értékeitől.



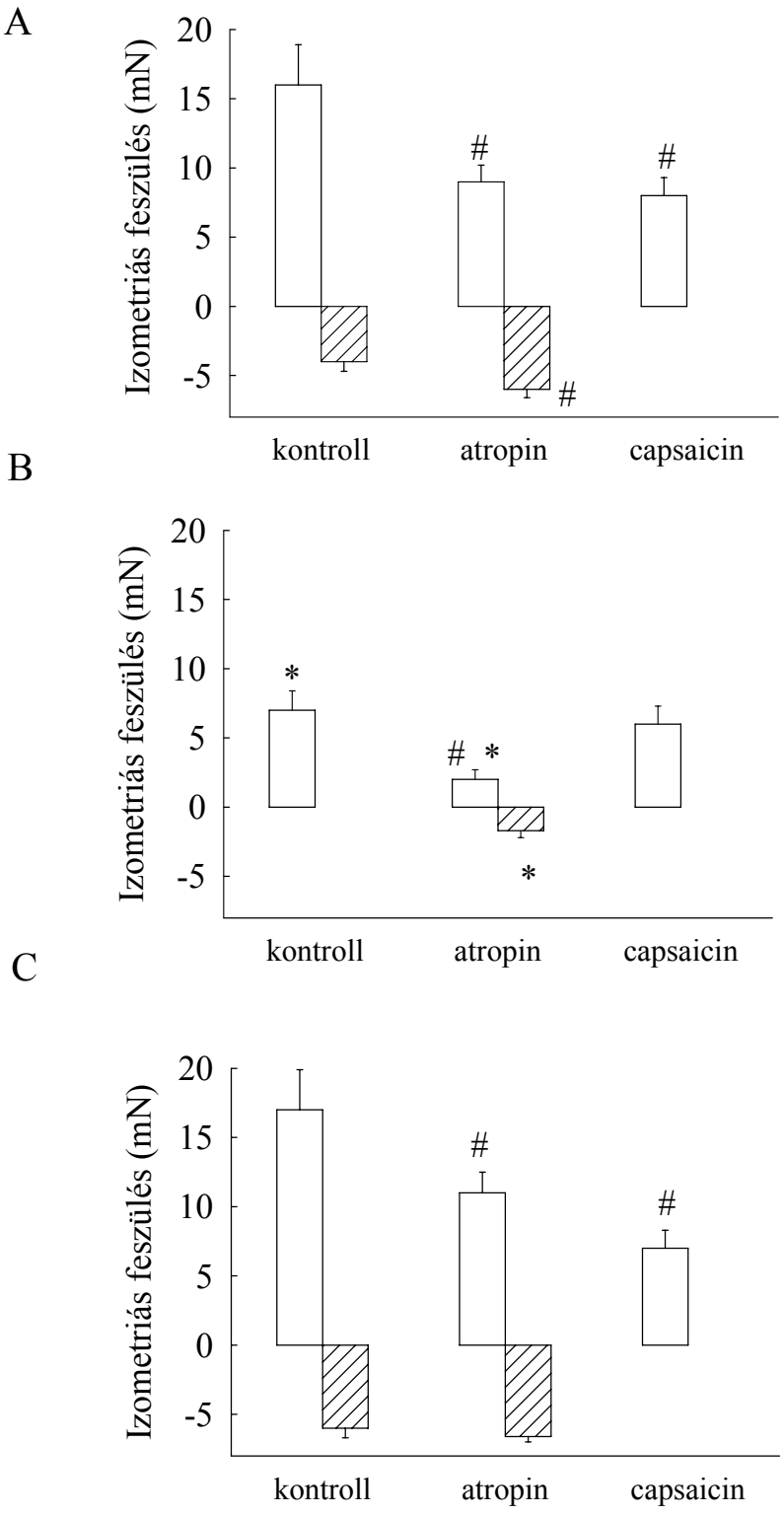
20. ábra N. saphenuson mért ingerületvezetési sebesség mérése. Diabétesz- indukálta ingerületvezetési sebesség csökkenés a n. saphenus gyors, mielinhüvelyes „A"(bal panel), és lassú vezetésű mielinhüvely mentes „C" (jobb panel) rostjain. A méréseket 8 héttel az. intravénás STZ injekció (egyszeri, 50 mg/kg) adása után végeztük el. Az adatok átlagát ± S.D. -val fejeztük ki, egy csoportban 6 állat volt. *: a kontrolltól a kezelt szignifikánsan különbözött, $p \leq 0,05$.

13.5 Téringerléssel kiváltott izometriás tenzióváltozások

A kontroll állatokból származó bronchusgyűrűk TI bronchokonstrikciója bifázikus, azaz a kontrakciót relaxáció követi. A diabéteszes állatok téringerlés indukálta kontrakciója a kontrollhoz képest megváltozott. A TI kontrakció csökken, a relaxáció fokozódik. 10 perces TTX-el való előkezelés után a bronchusgyűrűk téringerlés hatására nem reagáltak.
Téringerlés hatására kialakult kontrakciók mind atropin (1µM), mind capsaicin inkubáció hatására csökkent mértékben jöttek létre. Diabéteszes patkányokból származó gyűrűk téringerlés hatására gyengébben kontraháltak a normálhoz képest. Capsaicin előkezelés számottevően nem befolyásolja a TI bronchokonstrikciót. Atropin kezelés hatására diabéteszben a TI bronchorelaxáció gyengül. Az inzulin inplantátumot kapott állatok bronchuspreparátumai téringerlés hatására a kontroll állatok mintáihoz hasonlóan reagáltak (21.ábra).



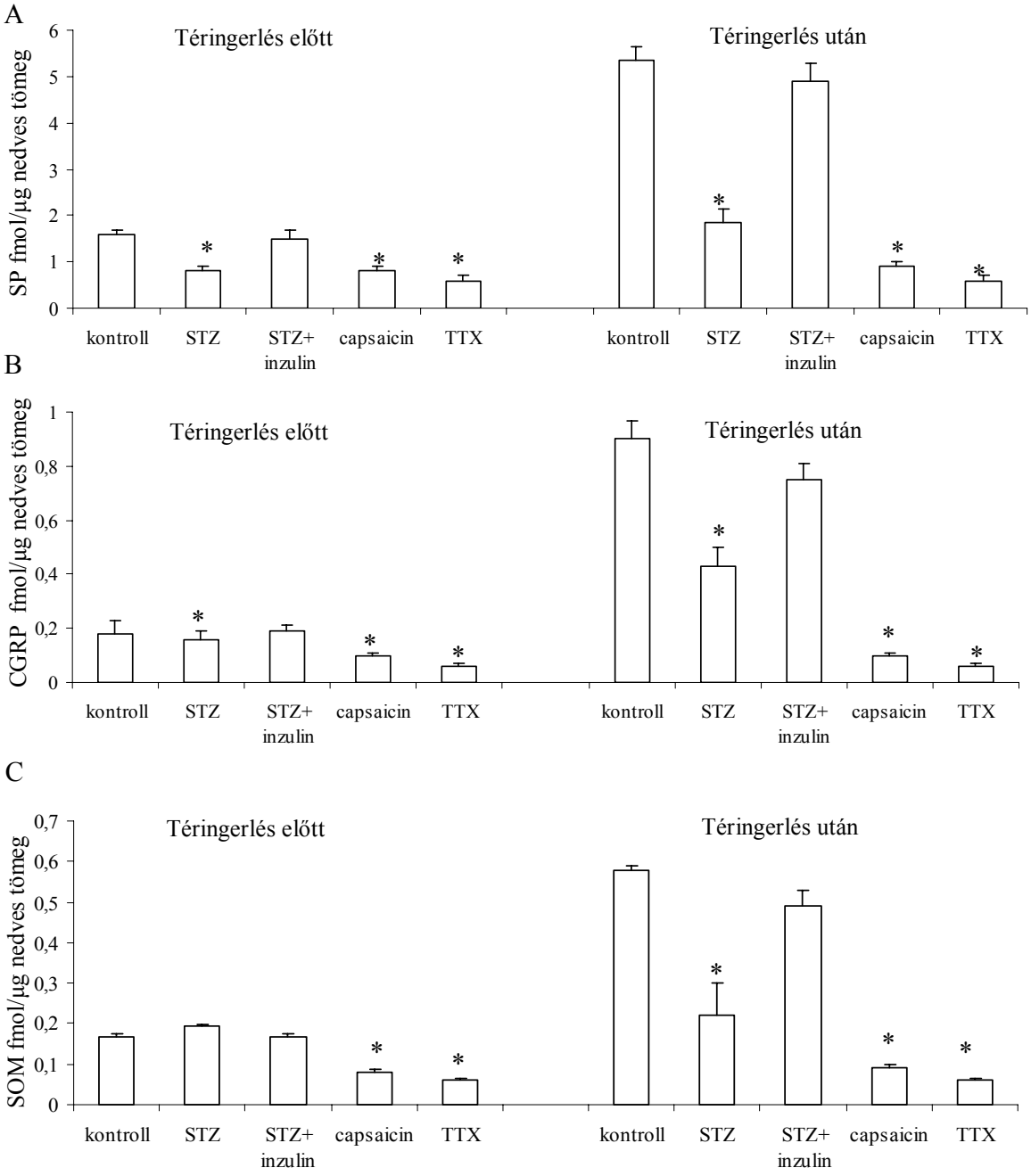
21. ábra: Eredeti regisztrátumok mutatják a kontroll (A, B, C panelek) és a diabéteszes (D, E, F panelek) állatokból származó bronchusgyűrűk TI bronchomotilitás változásait. A téringerlés paraméterei a következők voltak: 20V, 100 stimulus, 0.1 ms, 20Hz. A szervfürdőbe közvetlenül adtuk az 1μM atropint. A capsaicin-szenzitív „C" rostokat -szelektíven- sorozatos capsaicin előkezeléssel károsítottuk. „A"és „D" paneleken kezelés nélkül, TI bronchomotilitás változás; „B" és „E" paneleken: atropin hozzáadása után a TI bronchomotilitás változás; „C" és „F" paneleken: capsaicin előkezelés után a TI bronchomotilitás változás látható.



22. ábra TI bronchomotilitás változás adatok átlagainak összefoglalása. „A" panel: Az adatok átlagait ± S.D.-val fejeztük ki. Csoportonként 6 állatot használtunk fel.*: Szignifikáns különbség a diabéteszhez képest. #: Szignifikáns különbség a kontrollhoz képest. A téringerlés paraméterei: 20V, 100 stimulus, 0.1 ms, 20 Hz voltak. A előfeszítés 15mN volt. *,#: $p \leq 0.05$. „A" panel: kontroll; „B": diabéteszes; „C" diabéteszes inzulinnal kezelt állatok bronchomotilitás változásai.

13.6 Tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek vizsgálata diabéteszben

Téringerlés (100 stimulus, 20V, 0.1 ms, 20 Hz) hatására patkány tracheából felszabaduló, SOM CGRP, SP mennyiségét szervfürdőből RIA-val határoztuk meg. A kontroll csoport tracheáiból téringerlés hatására felszabaduló SOM CGRP és SP a kezdeti 0.17 ± 0.0022, 0.15 ± 0.0022 és 1.65 ± 0.093-ról 0.58 ± 0.032, 0.74 ± 0.122 és 5.34 ± 0.0295 fmol/mg nedves tömegre változott. A diabéteszes csoport tracheáiból téringerlés hatására felszabaduló SOM, CGRP és SP 0.19 ± 0.016, 0.11 ± 0.019, és 0.98 ± 0.116-ról 0.22 ± 0.076, 0.34 ± 0.099 és 1.84 ± 0.316 fmol/mg nedves tömegre csökkent.Az inzulin inplantátumot kapott állatok tracheáiból felszabaduló szenzoros neuropeptidek koncentrációváltozása a kontrollhoz volt hasonló (23. ábra).

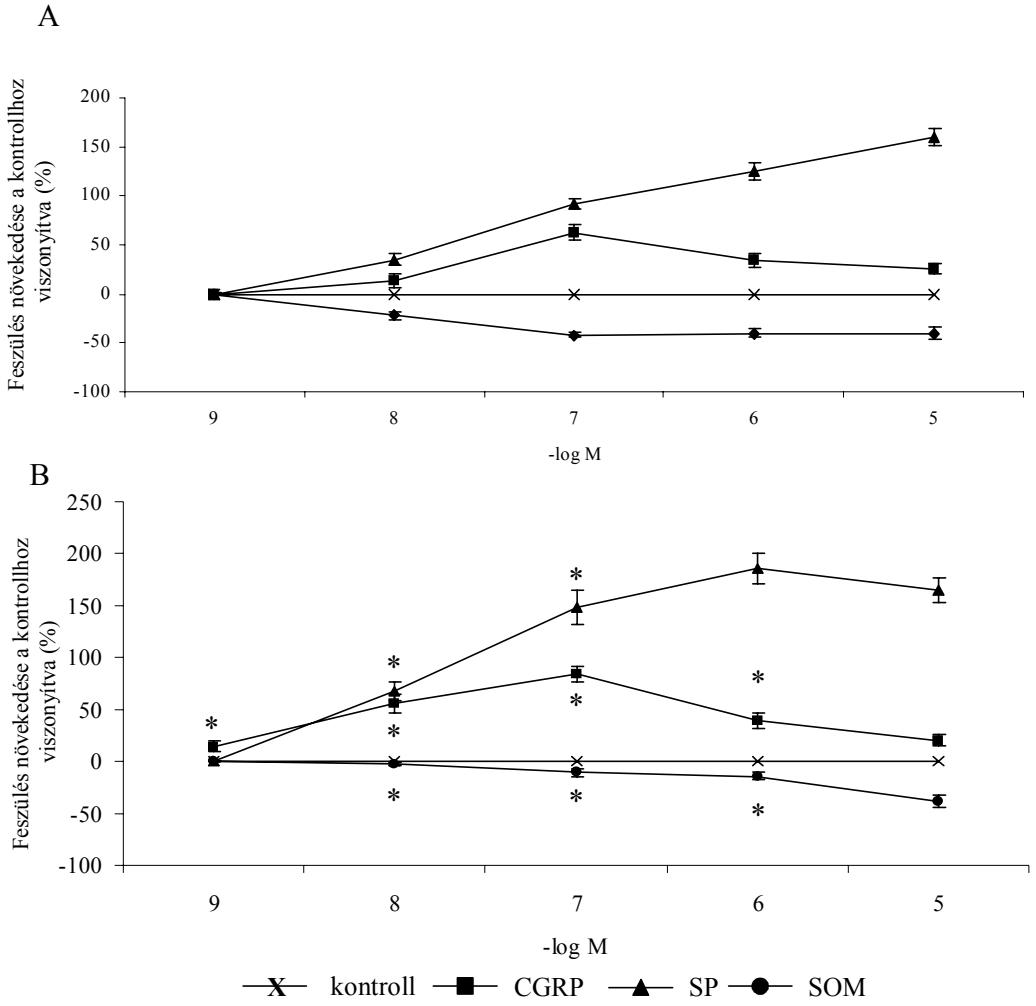


23. ábra Téringerlés hatására tracheából felszabaduló szenzoros neuropeptidek változása. A tracheák kontroll, diabéteszes, diabéteszes és inzulin inplantátutmot kapott, és capsaicinnel előkezelt állatokból származtak. TTX-al előkezelt tracheákból téringerlés hatására nem szabadult fel neuropeptid. A mintavételek a téringerlés előtt (bal oldal), közvetlenül a téringerlés után (jobb oldal) történtek. A téringerlés paraméterei a következők voltak: 20 V, 100 stimulus, 0.1 ms, 20 Hz. Az adatok átlagát ± S.D.-val fejeztük ki. A méréseket csoportonként 6 állaton végeztük el. *: A kontrollhoz képest szignifikáns különbség, $p \leq 0.05$.

13.7 A szenzoros neuropeptidek hatása a TI bronchokonstrikcióra

Mechanikusan előfeszítettük a kontroll és a diabéteszes állatokból származó bronchusgyűrűket. Megvizsgáltuk az exogén neuropeptidek (SOM, CGRP, SP) bronchokonstrikcióra gyakorolt hatását. Az egészséges és a diabéteszes állatok bronchusainak bronchokonstrikcióját sem a SOM, sem a CGRP nem befolyásolta, viszont az SP koncentrációfüggő módon növelte. Az exogén SP által kiváltott maximális bronchokonstrikciók értékei a következők voltak: kontrol állatoknál: 12.3 ± 2.7 mN –logEC_{50} 7.1 ± 0.2–el, míg diabéteszes állatoknál: 13.6 ± 3.4 mN :–logEC_{50} 7.0 ± 0.1–el. Amikor az exogén SP TI bronchomotilitásváltozásra gyakorolt hatását vizsgáltuk a bronchusok előfeszülését mindig vissza kellett álltani 12 mN-ra.

A CGRP (0.1 μM-ig) és a P-anyag (1 μM-ig) növeli mind a normál, mind a diabéteszes állatokból származó gyűrűk TI bronchokonstrikcióját. A neuropeptidek potencírozó hatása a TI indukálta bronchokonstrikcióra kifejezettebb a diabéteszes állatokból származó bronchusgyűrűkön, mint a kontrollokén. Az exogén SOM mind a kontroll, mind a diabéteszes csoportban gyengítette a TI bronchokonstrikciót, de diabéteszben a gyengülés a kontrollhoz képest kifejezettebb volt.

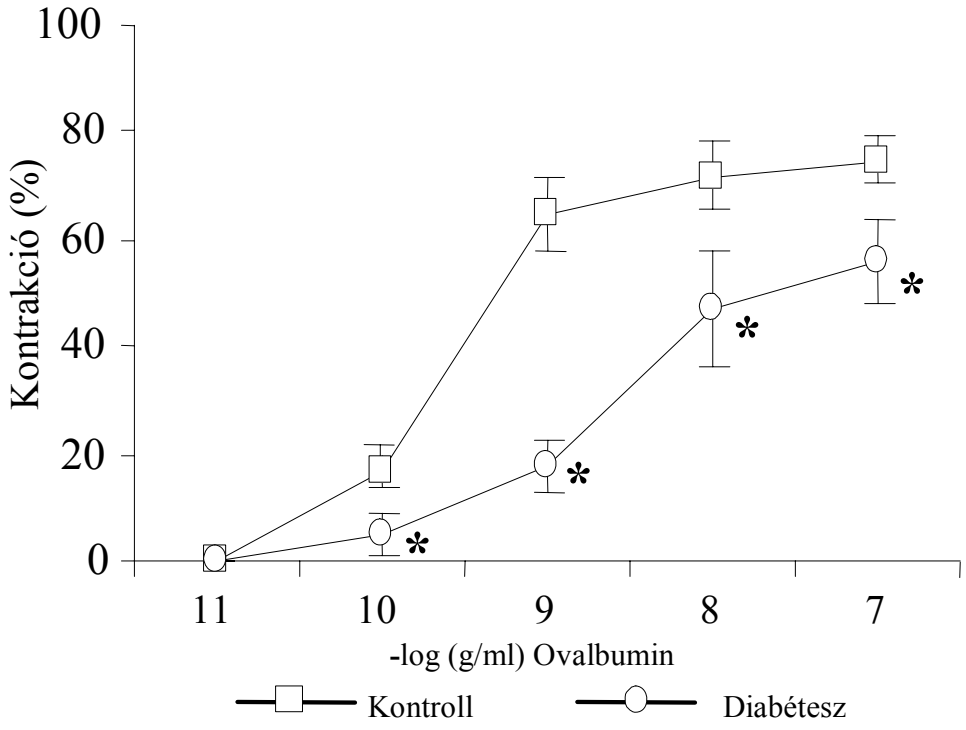


24. ábra Exogén CGRP, SP és SOM TI bronchokonstrikcióra gyakorolt hatása. „A" panel: kontroll csoport, „B" panel: diabéteszes csoport. Az adatok átlagait ± S.D.-val fejeztük ki. Csoportonként 6 állatot használtunk fel. A vízszintes vonal a 0%-nál jelzi mind a kontroll, mind a diabéteszes állatokból származó gyűrűk téringerlés hatására exogén neuropeptid kezelés nélkül kialakult bronchokonstrikciót. A pozitív értékek az exogén CGRP, SP kezelés hatására kialakult kontrakció növekedését jelzik, míg a negatív értékek SOM hatására kialakult bronchokonstrikció csökkenését jelzik. A téringerlés paraméterei: 20 V, 100 stimulus, 0.1 ms, 20 Hz voltak. A előfeszülés 12 mN volt. *: A kontrollhoz képest szignifikáns különbség: $p \leq 0.05$.

13.8 Antigén indukálta trachea kontrakció ovalbuminnal szenzitizált tengerimalacban

Az ovalbuminnal szenzitizált nem-diabéteszes tengerimalacokból származó trachea gyűrűk ovalbumin hatására, ovalbumin koncentrációjától függően kontrahálnak. Az ovalbumin koncentrációját logaritmikus egységenként növeltük. Az ovalbuminnal kiváltott maximális kontrakciók értékei ≈70%-a az 1 mM carbacholnak. Az ovalbumin kezelés hatására létrejövő bronchokontrakciós válaszok mértéke fokozódott, a kapott koncentráció-

hatás görbe jobbra tolódott. Az ovalbumin indukálta kontrakciók EC_{50} értékei a következők voltak: kontroll állatok esetén $4x10^{10}$ és diabéteszes állatok esetén $6x10^{9}$ g/ml. Az ovalbumin indukálta bronchokonstrikció maximuma diabéteszes állatokban szignifikánsan csökkent a kontrollhoz képest.



25. ábra Ovalbuminnal kiváltott bronchokonstrikció. A bronchusgyűrűk ovalbuminnal szenzitizált kontroll és diabéteszes tengerimalacokból származtak. Az adatokat a referens kontrakció (1mM carbachol indukálta kontrakció) százalékában adtuk meg. Az adatok átlagait ± S.D.-val fejeztük ki. Csoportonként 6 állatot használtunk fel.*: ovalbumin indukálta bronchokonstrikció diabéteszes állatban szignifikánsan különbözik a kontrolltól, $p \leq 0.05$.

14 EREDMÉNYEK RÖVID MEGBESZÉLÉSE

Megfigyeléseink szerint a cisplatin ill. STZ kezelés indukálta experimentális szenzoros neuropátiában megváltoztak a bronchusokat innerváló szenzoros rostok szenzoro-effektor funkciói.

Mindkét általunk alkalmazott experimentális szenzoros neuropátia modellben a téringerlés indukálta NANC bronchokonstrikció csökkent, míg a NANC bronchorelaxáció amplitúdója nőtt. A jelenség hátterében a szenzoros idegek szenzoros effektor funkcióváltozása áll. Cisplatin indukálta szenzoros neuropátiában fokozódik a szabadgyökök és a nitrogén – monoxid termelése, ami peroxi-nitrit képződésen keresztül növeli a téringerlés indukálta NANC bronchorelaxáció amplitúdóját és hosszát. RIA tanulmányok szerint cisplatin-, valamint STZ- indukálta szenzoros neuropátiában a téringerlés indukálta NANC bronchokonstrikció csökkenés együtt jár a tracheából téringerlés hatására felszabaduló szenzoros neuropeptidek (CGRP, SP és SOM) felszabadulásának csökkenésével. Inzulin inplantátum hatására a tracheából téringerlés indukálta szenzoros neuropeptid felszabadulás normalizálódik. Eeen experimentális szenzoros neuropátiában megfigyelt mechanizmus, azaz a szenzoros neuropeptidek felszabadulásának csökkenése terápiás előnyökkel járhat olyan betegségeknél, mint pl. az asztma bronchiale.

A RIA vizsgálatok szerint mind cisplatin, mind STZ kezelés hatására krónikusan növekszik a plazma SOM szint. A szenzoros neuropátiában a plazma SOM színtjének változása – legalábbis a fenti eredmények ismeretében- központi jelentőségű.

A cisplatin indukálta szenzoros neuropátiában megfigyelt plazma SOM krónikus SSTR4 mRNS overexpressziót okoz a bronchus mukóza alatti szöveteiben. A cisplatin kezelés ezért előnyös lehet bizonyos endogén SSTR-t expresszáló tumorok gyógyításában.

Diabéteszben az exogén CGRP, SP a kontrollhoz képest fokozta a bronchokonstrikciót, míg a SOM bronchokonstrikciót kevésbé gyengítette. Ovalbuminnal szenzitizált állatokban az ovalbumin indukálta bronchokonstrikció a kontrollhoz képest diabéteszben szintén csökkent.

Diabéteszben a plazma SOM koncentráció növekedése deszenzibilizálja a bronchusokat, csökkentve a bronchusok hiperreaktivitását. A bronchusok hiperreaktivitásának ezen feltételezett mechanizmuson keresztüli csökkenése az asztma bronchiale gyógyításában szintén terápiás értékű lehet.

15 ÖSSZEFOGLALÁS

Idegkárosodás (neuropátia) előfordulhat önálló kórképként, de kialakulhat betegségek, különböző kezelések mellékhatásaként is. Megkülönböztetünk szenzoros, autonóm, ill. motoros neuropátiákat aszerint, hogy a funkciózavar mely idegeken domináns.

Experimentális körülmények között vizsgáltuk funkcionális következményeit a cisplatin valamint a diabetesz indukálta szenzoros neuropátiának, amely kísérletekből származó eredményeknek közvetlen klinikai vonatkozásai lehetnek.

A szenzoros effektor funkcióváltozásokat téringerlés indukálta (TI) bronchomotilitás vizsgálatok segítségével, a tracheából téringerlés hatására felszabaduló, valamint a plazma szenzoros neuropeptidek (calcitonin gén –rokon peptid (CGRP), P-anyag (SP) és szomatosztatin (SOM)) mennyiségének változását radioimmunoassay-vel (RIA) határoztuk meg. A calcitonin gén –rokon peptid receptor (CGRPR) neurokinin1 (NK_1), neurokinin2 (NK_2), szomatosztatin receptor 4 (SSTR4) mRNS-eit quantitativ real time – polymerase chain reaction-nal (QRT-PCR) vizsgáltuk. A kísérleteket hím Wistar patkányokon és Dunkin – Hartley tengerimalacokon végeztük el.

Az általunk vizsgált modellekben (cisplatin, diabetes) a TI non – adrenerg, non – cholinerg (NANC) bronchokonstrikció csökkent, relaxáció fokozódott. Diabéteszben valamint a cisplatin indukálta szenzoros neuropátiában megnövekedett plazma SOM szintet tapasztaltunk, míg mind a diabéteszes, mind a cisplatinnal kezelt állatokból származó bronchusokból csökkent SOM felszabadulást észleltünk téringerlés hatására, miközben az ovalbuminnal kiváltott bronchiális hiperreaktivitás csökkent. Cisplatin indukálta szenzoros neropátiában a bronchusok SSTR4 mRNS-ének a transzkripciós mintázata megváltozott, a vizsgált periódus alatt krónikusan növekedett. A többi vizsgált receptor (CGRPR, NK_1, NK_2) expressziós mintázata a kezelést követő 11. napon fokozódott, a 22. napon azonban a kontroll szintre tért vissza.

Az experimentális szenzoros neuropátiában megfigyelt téringerlés indukálta NANC bronchokonstrikció gyengülés hátterében a felszabaduló szenzoros neuropeptidek csökkenése áll. Ez a mechanizmus, valamint a diabéteszben megfigyelt hiperszomatosztatémia hozzájárulhat a bronchiális hiperreaktivitás csökkenéshez, mely mechanizmus terápiás értékű lehet az asztma bronchiale gyógyításában. A cisplatin indukálta hiperszomatosztatémia és az SSTR4 overexpresszió miatt a cisplatin terápia előnyös lehet bizonyos endogén, szomatosztatin receptort expresszáló tumorokban.

16 SUMMARY

Neuropathy is a common disorder, which may be induced by various illnesses and may develop as a side effect of medical treatments. Neuropathy involves the autonomic and/or the sensory nervous system.

The effector function of the sensory neurons were examined after diabetic neuropathy induction or cisplatin induced neuropathy. The results of the experiments may have direct clinical evidences.

The changes in sensory effector function were examined by field stimulation (FS) induced bronchomotility studies; FS released sensory neuropeptide (calcitonin gene –related protein (CGRP), somatostatin (SOM) from trachea, and plasma level of CGRP, SP, SOM by radioimmunoassay; changes in transcription pattern of mRNAs of neurokinin1 (NK_1), neurokinin2 (NK_2), somatostatin receptor 4 (SSTR4) by quantitative real time – polymerase chain reaction. Male Wistar rats and Dunkin – Hartley guinea pigs were used for the experiments.

Field stimulation-induced non–adrenergic, non–cholinergic (NANC) bronchoconstriction was significantly attenuated in rings from animals suffered from sensory neuropathy, however the amplitude of the relaxation phase was significantly attenuated in cisplatin–induced sensory neuropathy.

FS induced increase in CGRP, SOM, SP release from tracheae was significantly attenuated in preparations from animals with sensory neuropathy. Fasting plasma SOM level significantly increased in diabetic/cisplatin treated animals. Cisplatin treatment changed the expression pattern of the sstr4, and induced significant overexpression in the SSTR4 mRNA.

It is concluded, that the increased SOM level caused desensitization which may develop to the effect of somatostatin in rings from diabetic rats, thus leading to decrease in bronchial hyperreactivity. The attenuated bronchomotor response is related to a decrease in sensory neuropeptide release in experimental sensory neuropathy, at least in our conditions.

This change in sensory–effector function and SOM induced decrease in bronchial hyperreactivity may have clinical evidences in treatment of bronchial asthma. Taken the SSTR4 overexpression together with hypersomatostatinaemia it is suggested that cisplatin might be of particular importance as a therapeutic tool in patients with endocrine tumors.

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21 FÜGGELÉK

21.1 Impaired bronchomotor responses to field stimulation in guinea-pigs with cisplatin-induced neuropathy

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21.1.1 *Abstract*

Pre-treatment with cisplatin (3 mg/kg) i.p. once a day over 6 days induced sensory neuropathy as confirmed by femoral nerve conduction velocity test and significantly decreased contractions induced by electrical field stimulation (100 stimuli, 20 V, 0.1 ms, 20 Hz) in isolated main bronchial rings from guinea-pigs. The field stimulation-induced non-adrenergic, non-cholinergic (NANC) relaxations, however, were amplified in rings from animals with cisplatin neuropathy. The NANC relaxation response was completely blocked by 30 μM N^G-nitro-L-arginine methyl ester in preparations from both control and cisplatin-treated animals. Superoxide dismutase (40 units/ml) was without effect on NANC relaxation in control rings, however, it substantially decreased NANC relaxation in preparations from animals with cisplatin neuropathy. These results show that cisplatin-induced sensory neuropathy is accompanied by attenuation of neural bronchoconstriction and an enhanced NANC relaxation. The latter is in part attained by an increased peripheral superoxide production.

Author Keywords: Cisplatin; Sensory neuropathy; Superoxide production; Peroxinitrite

21.1.2 *Article Outline*

21.1.3 *Introduction*

Capsaicin-sensitive primary afferent neurons have been shown to play a major modulatory role in the regulation of bronchomotility (Coburn and Barnes). Regarding the neurotransmitters involved, most evidence favours a role for calcitonin gene-related peptide (CGRP), substance P, neurokinin A, somatostatin and possibly nitric oxide (NO) (Nijkamp and Szolcsanyi). It is therefore not surprising that diseases or pharmacologic manoeuvres result in the damage of these neurons such as progression of Type I diabetes or treatment with capsaicin impair bronchomotility (Helander; Casaco and Perretti).

Cisplatin is a chemotherapeutic agent used for the treatment of several types of cancer. Unfortunately, cisplatin's therapeutic potential is limited by divers adverse effects such as myelosuppression, nephrotoxicity, ototoxicity and neurotoxicity (Stewart; Fillastre and Ozols). The drug-induced neurotoxicity is characterized by a decrease in sensory nerve conduction velocity and a deficiency in axonal transport of sensory neuropeptides such as that of CGRP, substance P, galanin and somatostatin (Barajon et al., 1996). However, to the best of our knowledge, no study has been conducted as to whether a sensory-effector dysfunction occurring in cisplatin-neuropathy is reflected in changes in bronchial reactivity. The present work was therefore concerned with the possibility that neurotoxic cisplatin doses may deteriorate non-adrenergic, non-cholinergic (NANC) contractile responses to field stimulation in isolated bronchial preparations of the guinea-pig.

21.1.4 *Methods*

21.1.4.1 Ethics

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of Medical Universities of Pecs and Debrecen, Hungary.

21.1.4.2 Treatment groups

The study was carried out with 15 male guinea-pigs weighing 350–400 g. They were housed in an animal room (12-h light/dark periods a day, temperature of 22–25°C, humidity of 50–70%) with two animals per pen fed commercial laboratory chow and tap water ad libitum. The animals were random divided into two experimental groups. Control: animals treated with the solvent for cisplatin (1 ml isotonic NaCl) with 75 mg/kg mannitol i.p. once a day

over 6 days; Cisplatin-treated: animals treated with 3 mg/kg cisplatin with 75 mg/kg mannitol i.p. once a day over 6 days.

21.1.4.3 Isometric tension measurements

After exsanguination, rings from the main bronchi (3 mm) were mounted horizontally on two small L-shaped glass hooks of which one was connected to a force transducer for measurement of isometric tension. The experiments were carried out in thermostatically controlled (37±0.2°C) organ bath (5 ml) (TSZ 02, EXPERIMETRIA UK, London, England) containing Krebs solution. The organ fluid was gassed with 95% O_2 and 5% CO_2 to maintain pH at 7.40±0.05. Neural effects on contractile activity of the segments were studied by means of field stimulation (100 stimuli at 20 V, 0.1 ms and 20 Hz at an initial tension of 15 mN). The rings were prepared from six animals in each group. To study whether the field stimulation protocol applied was selective for nerve-mediated responses, some rings underwent a period of 10-min pre-incubation with tetrodotoxin, a fast sodium channel blocker. Stimulation with these parameters failed to elicit any contractile response in the presence of 1 μM tetrodotoxin.

21.1.4.4 Nerve conduction velocity studies

This series of experiments was carried out to verify/exclude sensory neuropathy. Left saphenous nerve conduction velocity was determined in animals from both groups as described (Nemeth et al., 1999b). In brief, in artificially ventilated animals anaesthetized with sodium thiopentone (30 mg/kg i.p.) the nerve was prepared, cleaned of fat ad adhering connective tissues and strains of square-wave (500 μs) constant voltage stimuli were applied through pairs of platinum electrodes (EXPERIMETRIA UK) placed as high as possible. Another pair of electrodes was applied 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding 'A' and 'C' signals were determined for calculation of average conduction velocity by dividing the inter-electrode distance by the interval between the end of the stimulatory impulse and the appearance of the 'A' and 'C' signals (Janig and Lisney, 1989).

21.1.4.5 Experimental protocol

The animals in each group were anaesthetized for nerve conduction velocity studies 24 h after the last cisplatin/vehicle dose. Bronchial rings were then prepared from the same animals for isometric tension measurements. After a 60-min period of equilibration, the rings were subjected to the field stimulation protocol. Two series of stimuli were applied to study the reproducibility of the responses. The rings were then incubated with guanethidine (4 μM) and atropine (1 μM, 'NANC' solution) and the field stimulation protocol was repeated. Subsequently, the preparations were exposed to additional incubation with 30 μM N^G-nitro-L-arginine methyl ester, a NO synthase inhibitor (Rees et al., 1990) followed by the field stimulation manoeuvre again. After completion of these interventions, the preparations were extensively rinsed until the initial contractile responses to field stimulation were re-gained. Separate rings were used for studies with capsaicin and superoxide dismutase, respectively. In order to determine whether capsaicin-sensitive sensory neurons contributed to the electrically evoked contractile responses, the rings were exposed to capsaicin (100 μM) over a period of 30 min after the control stimulation had been accomplished. This was followed by a 30-min washout period and the stimulation protocol was applied again. To study whether removal of

the superoxide anions influenced the NANC contractile responses, rings from both groups incubated in NANC solution were exposed to the field stimulation protocol in the presence or absence of superoxide dismutase (40 units/ml).

21.1.4.6 Drugs and chemicals

All drugs and chemicals applied have been purchased from Sigma (St Louis, Mo) except capsaicin which was from Fluka, Buchs, Switzerland). Guanethidine, atropine and N^G-nitro-L-arginine methyl ester were dissolved in Krebs solution, capsaicin was dissolved in ethanol diluted with Krebs solution.

21.1.4.7 Statistics

The results expressed as means±S.D. were analyzed with one-way analysis of variance followed by a modified *t*-test for repeated measures according to Bonferroni's method (Wallenstein et al., 1980). Changes were considered significant at $P<0.05$.

21.1.5 *Results*

21.1.5.1 Exclusions

Four cisplatin-treated animals had to be excluded from the experiments, two of them as cisplatin failed to produce any decrease in nerve conduction velocity in either A or C fibres, one because of respiratory insufficiency due to pneumonia and one because of the development of extended skin lesions.

21.1.5.2 Body weight and rectal temperature

Body weight decreased from pre-treatment value of 381±41 to 308±31 g ($P<0.05$) in the cisplatin-treated animals. The 'control' guinea-pigs did not exhibit any change in body weight during the treatment period. Rectal temperature did not change in animals in either group.

21.1.5.3 Nerve conduction velocity

Fig. 1 shows the cisplatin-induced decrease in nerve conduction velocity in fast conducting myelinated (A fibres in Fig. 1, left panel) and slow conducting unmyelinated (C fibres in Fig. 1, right panel) fibres. At a stimulation intensity suprathreshold for A (0.5 V, 5 Hz) or C (3 V, 5 Hz) fibres, conduction velocity significantly decreased in cisplatin-treated animals.

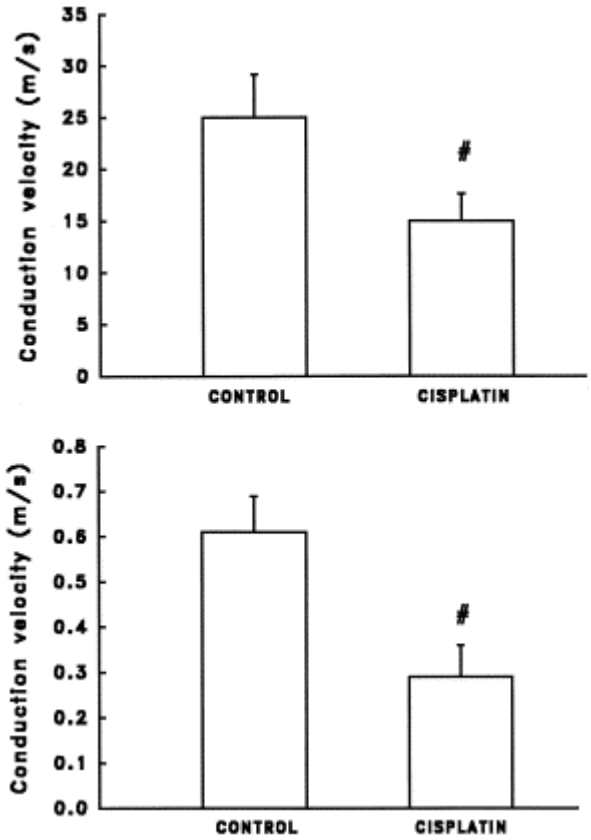
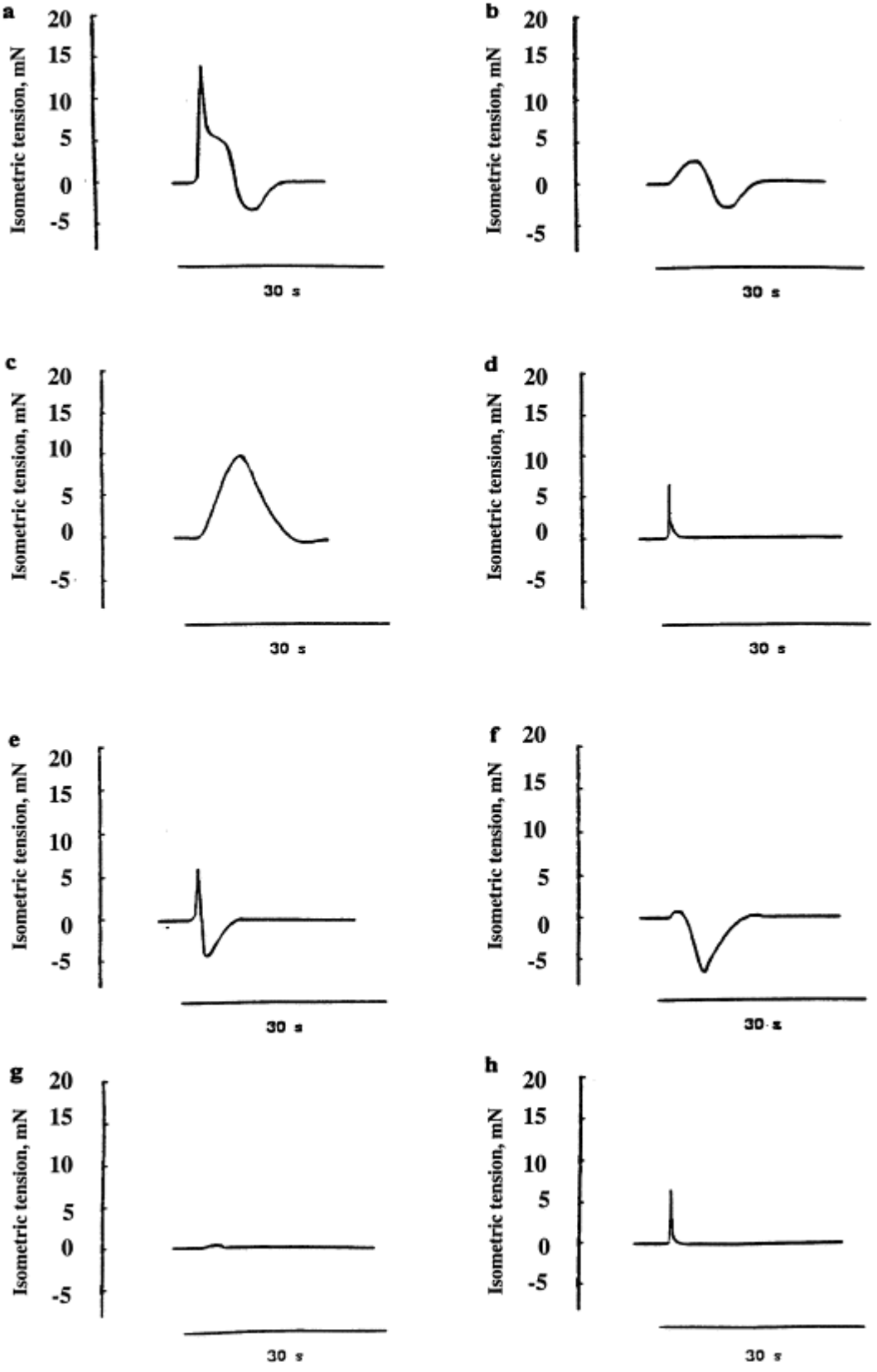


Fig. 1. Cisplatin-induced decrease in nerve conduction velocity in fast conducting myelinated (left panel) and slow conducting unmyelinated C (right panel) fibres of the femoral nerve. Measurements were done 24 h after a series of six intraperitoneal injections of cisplatin (3 mg/kg) and/or its vehicle once a day. The data are means±S.D. obtained with six animals per group. #: significantly different from control at $P<0.05$.

21.1.5.4 Changes in isometric tension in response to field stimulation

Field stimulation in tracheal rings from 'control' animals evoked a biphasic response, the first contractile component of which comprised an initial fast and a subsequent slow reaction. This two-phase contraction was followed by a relaxation response. The fast contractile component was abolished in NANC solution, whereas the slow one disappeared in tissues pre-exposed to 100 μM capsaicin. The NANC relaxation was blocked after a 30-min incubation with 30 μM N^G-nitro-L-arginine methyl ester (Fig. 2).

A



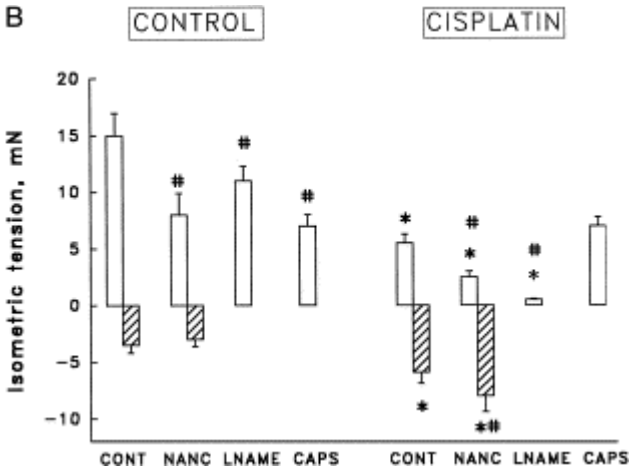


Fig.2. Original tracings (A) and summarizing data (B) representing changes in isometric tension in bronchial preparations from control (first and second rows) and cisplatin-treated (third and fourth rows) guinea-pigs in response to field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli); (a) rings from control animals in Krebs solution, (b) rings from control animals incubated in 'NANC' (4 μM guanethidine and 1 μM atropine) solution, (c) 'NANC' solution plus 30 μM N^G-nitro-L-arginine methyl ester (L-NAME), (d) after capsaicin (100 μM), (e) rings from cisplatin-treated animals in Krebs solution, (f) rings from cisplatin-treated animals incubated in 'NANC' solution, (g) rings from cisplatin-treated animals in 'NANC' solution plus 30 μM L-NAME; h) rings from cisplatin-treated animals after capsaicin (100 μM). In part B, open columns represent contractions, hatched columns show relaxations. The data are means±S.D. obtained with six preparations per group. #: significantly different from CONT (Krebs solution only) at $P<0.05$; * control vs. corresponding cisplatin-treated at $P<0.05$. In rings from animals treated with cisplatin, both the amplitude and duration of the field stimulation-induced contractile phase were significantly attenuated. However, the amplitude of the relaxation phase sensitive to N^G-nitro-L-arginine methyl ester was augmented. The contractile 'spike' left by pre-incubation with capsaicin, however, was similar in preparations from either group (Fig. 2).

The NANC contractile responses were not influenced by superoxide dismutase in preparations from the control animals. In preparations from the cisplatin-treated group, however, the NANC relaxation phenomenon was substantially reduced in the presence of superoxide dismutase (Fig. 3).

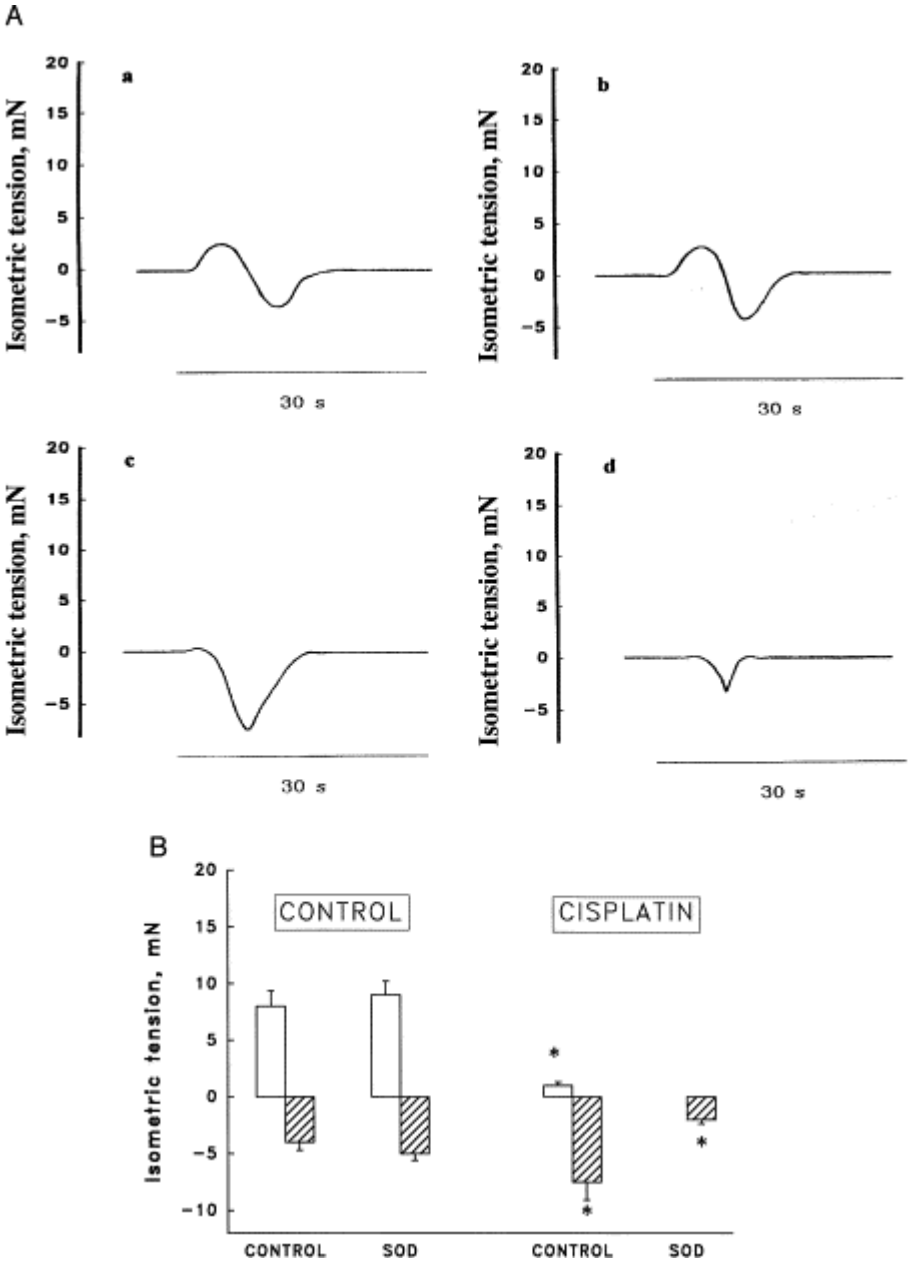


Fig. 3. Original tracings (A) and summarizing data (B) indicating changes in tension in bronchial rings from control (first row) and cisplatin-treated (second row) guinea-pigs in response to field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli). (a) NANC solution; (b) NANC solution with 40 units/ml superoxide dismutase; (c) rings from cisplatin-treated animals, NANC solution; (d) NANC solution with 40 units/ml superoxide dismutase. In part B, open columns represent contractions, hatched columns show relaxations. The data are means±S.D. obtained with six preparations per group. * control vs. corresponding cisplatin-treated at $P<0.05$.

21.1.6 *Discussion*

These results indicate that cisplatin-induced sensory neuropathy is associated with a reduced contractile response to field stimulation in isolated bronchi of the guinea-pig. This attenuated reaction can be attributed to a deficiency in sensory-effector nerve function since it was the atropine-resistant and capsaicin-sensitive section of the contractile response, which was

deteriorated by cisplatin. Nevertheless, the NANC relaxation was much more pronounced in preparations from the cisplatin-treated group than in those from the control animals. Since the NANC relaxation response was completely blocked by N^G-nitro-L-arginine methyl ester in either group, it seems to be essentially nitrergic in nature. However, the nitrergic relaxation response was significantly attenuated by scavenging the superoxide anions with superoxide dismutase in preparations from the cisplatin-treated animals contrary to that seen in 'control' rings in which superoxide dismutase was without effect.

The cisplatin-treated guinea pigs exhibited characteristic neurophysiological features of sensory neuropathy in that a significant decrease in conduction velocity occurred in both fast and slow conducting sensory nerve fibres. However, it has been found that the dorsal root ganglia cells serve as the primary targets for cisplatin to induce peripheral neuropathy (Gispen; Barajon and Cavaletti). It is therefore not surprising that sensory neuropathy highlights neurotoxicity produced by cisplatin (Gispen and Tredici). Neuromorphologic studies by Barajon et al. (1996) on cisplatin-induced changes revealed an accumulation of sensory neuropeptides (CGRP, substance P, galanin and somatostatin) in dorsal root ganglia with much more severe histological alterations in ganglionic cells than those seen in peripheral fibres. These studies also suggested an impaired axonal transport of sensory neuropeptides by cisplatin.

Bronchial preparations are known to be densely innervated by CGRP, substance P, neurokinin A and somatostatin containing unmyelinated afferents (Lundberg and Lundberg) which originate from the vagus nerve with cell bodies in the jugular, nodose and dorsal root ganglia (Springall et al., 1987). The sensory-effector function of these nerves is underlain by the release of these neuropeptides from the nerve terminals in response various challenges such as an increase in extracellular K^+ concentration, acidosis or electrical stimulation (Szolcsanyi; Ferdinandy and Szolcs). Since sensory neuropeptides play a major modulatory role in both NANC contractions and relaxations of the bronchial muscle, it is possible that a decreased availability of excitatory neuropeptides to be released by field stimulation is responsible for the feeble NANC contractions in preparations obtained from cisplatin-treated animals similar to neuropathy induced by insulin deficient diabetes (Nemeth et al., 1999a). However, the unexpected finding of the augmented nitrergic relaxation in rings from the cisplatin-treated group can hardly be explained on the basis of a decreased availability of transmitter/transmitter producing apparatus. Of course, attenuation of excitatory impulses may be of significant modifying effect but taking the difference in sensitivity of preparations from control or cisplatin-treated animals to superoxide dismutase into account, mechanisms other than simple negative synergism may be suspected. What is more likely is that NO release in response to electrical stimulation leads to peroxinitrite formation in the presence of increased amounts of superoxide anions (Beckman et al., 1990) the production of which is characteristic of cisplatin-induced neuropathy (Matsushima and Fukaya). This is suggested by the facts that NANC relaxation is completely blocked by NO synthase inhibition irrespective of the presence or absence of cisplatin neuropathy. On the other hand, the control preparations were resistant to superoxide dismutase, whereas the superoxide scavenger significantly attenuated the NANC response in rings from the neuropathic guinea-pigs.

In summary, the results presented show that cisplatin-induced sensory neuropathy is accompanied by attenuation of the effector function of sensory nerves regulating bronchial excitability. The decrease in contractile responses associated with an augmented NANC relaxation response possibly underlain by an increased peripheral superoxide production in the neuropathic animals. This calls attention to widespread alterations in neural regulatory

processes in cisplatin-induced neuropathy and to the possible benefit from cisplatin therapy in asthmatic patients suffering from neoplastic diseases.

21.1.7 *Acknowledgements*

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21.1.8 *References*

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21.2 Decreased sensory neuropeptide release in isolated bronchi of rats with cisplatin-induced neuropathy

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21.2.1 *Abstract*

We studied if attenuated neurogenic bronchoconstriction was associated with a change in sensory neuropeptide release in preparations from rats with cisplatin-induced neuropathy. Electrical field stimulation (100 stimuli, 20 V, 0.1 ms, 20 Hz) induced an increase in the release of somatostatin, calcitonin gene-related peptide (CGRP) and substance **P** determined by radioimmunoassay from baseline 0.18±0.01, 0.17±0.01 and 0.86±0.02, to 0.59±0.02, 1.77±0.04 and 5.96 fmol/mg wet tissue weight, respectively, in organ fluid of tracheal tubes from rats. This was significantly attenuated to post-stimulation values of 0.36±0.02, 0.45±0.02, 4.68±0.24 fmol/mg wet tissue weight for somatostatin, CGRP, and substance **P,** respectively, with a significant decrease in field stimulation-induced contraction of bronchial preparations from animals 11 days after a 5-day treatment period with cisplatin (1.5 mg/kg i.p. once a day). The cisplatin-treated animals developed sensory neuropathy characterized by a 40% decrease in femoral nerve conduction velocity. The results show that a decrease in tracheo-bronchial sensory neuropeptide release associates with feeble bronchomotor responses in rats with cisplatin-induced sensory neuropathy.

Keywords: Cisplatin; Sensory neuropathy; Neuropeptide release

21.2.2 *Article Outline*

21.2.3 *Introduction*

It has been shown that neurotoxic doses of cisplatin impair contractile responses to electrical field stimulation in isolated bronchial preparations of the guinea pig (Szilvassy et al., 2000). The attenuated neurogenic bronchoconstriction in cisplatin-induced neural dysfunction was found to be underpinned by altered non-adrenergic, non-cholinergic (NANC) responses, with a predominant deficiency in sensory-effector function of capsaicin-sensitive bronchial nerve fibres (Coburn and Tomita, 1973 and Barnes, 1990), similar to that found in association with diabetic neuropathy (Szilvassy et al., 2002). The sensory-effector function of these nerves is known to be executed by the release of sensory neurotransmitters in response to activation of sensory nerve terminals produced by various stimuli such as an increase in extracellular K^+ concentration, a decrease in tissue pH or electrical stimulation (Szolcsanyi, 1996 and Szolcsanyi et al., 1998). Regarding the neurotransmitters involved, most evidence favours an important role for sensory neuropeptides such as calcitonin gene-related peptide (CGRP), substance P, neurokinin A, somatostatin and possibly nitric oxide (NO) as an atypical neurotransmitter (Nijkamp and Folkerts, 1995 and Szolcsanyi, 1996). We therefore postulated that the cisplatin-induced reduction in bronchoconstrictory responses to field stimulation was associated with a decrease in bronchial sensory neuropeptide release.

21.2.4 *Materials and methods*

21.2.4.1 Ethics

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of the Universities of Pecs and Debrecen, Hungary.

21.2.4.2 Experimental groups and animals

The study was carried out with 20 Wistar male rats weighing 270–320 g. They were housed in an animal room 12-h light and dark periods a day, temperature of 22–25 °C, humidity of 50–70% with five animals per pen fed commercial laboratory chow and tap water ad libitum. The animals were randomized into two experimental groups. Control: animals treated with the solvent for cisplatin 1 ml isotonic NaCl with 75 mg/kg mannitol i.p. once a day over 5 days; Cisplatin-treated: animals treated with 1.5 mg/kg cisplatin (Bardos et al., 2003) with 75 mg/kg

mannitol i.p. once a day over 5 days. Body weight was measured each day over the experimental period.

21.2.4.3 Isometric tension measurements

Isolated segments of the main bronchi (2 mm) were mounted horizontally on two small L-shaped glass hooks of which one was connected to a force transducer for measurement of isometric tension. The experiments were carried out in thermostatically controlled (37±0.2°C) organ bath (5 ml) (TSZ 02, Experimetria UK, Hungary) containing Krebs solution (in mM): NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, KCl 4.7, $CaCl_2$ 2.5, glucose 11). The organ fluid was gassed with 95% O_2 and 5% CO_2 to maintain pH at 7.2±0.05. Neural effects on contractile activity of the segments were studied by means of field stimulation (100 stimuli at 20 V, 0.1 ms and 20 Hz at an initial tension of 12 mN). Eight rings were prepared from eight animals in each group. To investigate whether the field stimulation protocol applied was selective for nerve-mediated responses, some rings underwent a period of 10-min pre-incubation with tetrodotoxin, a fast sodium channel blocker, after the studies on neural effects on mechanical activity had been accomplished.

21.2.4.4 Neurotransmitter release studies

These have been described in detail elsewhere (Nemeth et al., 1999b). In brief, following exsanguinations, the lower third of the tracheae with the main bronchi were removed, cleaned of fat and adhering connective tissues. They were prepared for perfusion in a temperature (37 °C) and pH (7.2) controlled oxygenized Krebs solution over 60 min. Electrical field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli) was applied to elicit neurotransmitter release. Calcitonin gene-related peptide (CGRP), substance P, and somatostatin concentrations were determined from 200 µl samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories as described previously (Nemeth et al., 1999b). Determinations were done prior to as well as immediately and 2 min after field stimulation.

21.2.4.5 Nerve conduction velocity studies

This series of experiments was carried out to verify/exclude sensory neuropathy involving unmyelinated slow conducting ‘C’ fibres shown to play an important role in bronchomotility. Left saphenous nerve conduction velocity was determined in animals from both groups as described (Nemeth et al., 1999a, Nemeth et al., 1999b and Szilvassy et al., 2000). In brief, in artificially ventilated animals anaesthetized with sodium pentobarbital (80 mg/kg i.p.) the nerve was prepared, cleaned of fat and adhering connective tissues and strains of square-wave (500 µs) constant voltage stimuli were applied through pairs of platinum electrodes (Experimetria, UK) placed as high as possible. Another pair of electrodes was applied 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding compound ‘A’ and ‘C’ signals were determined for calculation of average conduction velocity. The inter-electrode distance was divided by the interval between the end of the stimulatory impulse and the appearance of the corresponding ‘A’ and ‘C’ signals (Janig and Lisney, 1989). The cisplatin-induced ‘C’ signal delay was used for characterization of cisplatin-induced sensory neuropathy.

21.2.4.6 Study design

The animals in either group were anaesthetized for femoral nerve conduction velocity studies 11 days after the last cisplatin/vehicle dose. After completion of these studies, the tracheae with the main bronchi were removed. Two-millimeter long segments from the main bronchi were used for isometric tension measurements, whereas the rest of the tissues were utilized for neuropeptide release studies. Schematic representation of the experimental protocol is seen in Fig. 1.

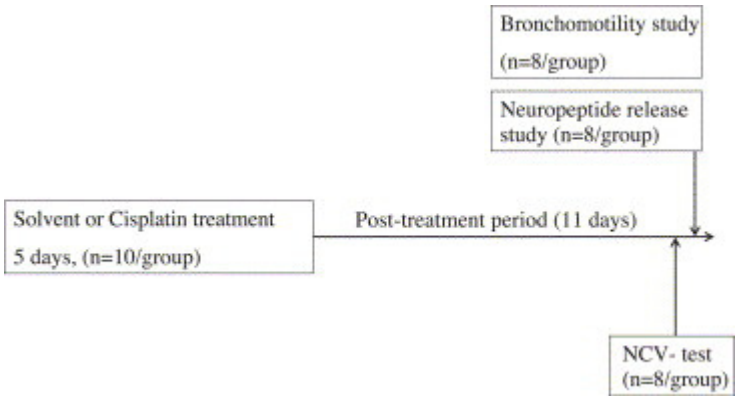

Fig. 1. Schematic representation of the experimental protocol applied. The animals randomized into two groups received either cisplatin (1.5 mg/kg/day i.p.) or its vehicle over 5 days. This period was followed by 11-day interval. The rats were then checked for the development of sensory neuropathy by means of femoral nerve conduction velocity studies. The animals were then sacrificed for in vitro bronchomotility investigations and bronchial neuropeptide release studies.

21.2.4.7 Drugs and chemicals

All drugs and chemicals used in this study were purchased from Sigma (St. Louis, USA), except cisplatin and its solvent, which were obtained from TEVA-BIOGAL (Debrecen, Hungary) and chemicals used for radioimmunoassay determinations, which were from sources as follows: [Tyr^1]-somatostatin-14 and Tyr-α-CGRP-(23–37) were purchased from Bachem (Bubendorf, Switzerland). The Subtance P RIA tracer was from Amersham (Little Chalfont, UK); Tween-80, NaH_2PO_4, Na_2HPO_4, NaCl and CH_2Cl_4 from Reanal (Budapest, Hungary); trifluoroacetic acid (TFA) and piperine from Fluka (Buchs, Switzerland); high pressure liquid chromatography (HPLC)-grade methanol from Carlo Erba (Rodano, Italy); Substance P antiserum was provided by Prof. G.J. Dockray, University of Liverpool, SOM and CGRP antiserum by Dr. T. Görcs, University Medical School of Budapest. Polypropylene RIA tubes (12×75 mm) were obtained from Merck (Darmstadt, Germany). ^{125}I-labelled Tyr-α-CGRP-(23–37) and ^{125}I-labelled [Tyr^1]-somatostatin-14 were prepared in our laboratory (Németh et al., 2002).

21.2.4.8 Statistical analysis

The isometric tension and nerve conduction velocity data expressed as means±standard deviation (S.D.) were evaluated with analysis of variance (ANOVA) followed by a modified *t*-test according to Bonferroni's method (Wallenstein et al., 1980). The blood chemistry data and sensory neuropeptide levels were evaluated by Student's *t*-test for unpaired data.

21.2.5 *Results*

21.2.5.1 Exclusions

Two cisplatin-treated animals had to be excluded from the experiments, one of them died, and the other did not show any evidence for the development of sensory neuropathy in response to the cisplatin treatment schedule applied.

21.2.5.2 Body weight and rectal temperature

Body weight decreased from pre-treatment value of 294±22 to 231±19 g (P<0.05) in the group of cisplatin-treated animals. The rats treated with the solvent for cisplatin exhibited a tendency to weight gain (306±20 vs. pre-treatment 289±23 g). Rectal temperature did not change during the experimental period in either group.

21.2.5.3 Nerve conduction velocity

Fig. 2 shows cisplatin-induced decrease in nerve conduction velocity in fast conducting myelinated (A fibres) and slow conducting unmyelinated (C) fibres. At a stimulation intensity suprathreshold for A- (0.5 V, 5 Hz) or C- (3 V, 5 Hz) fibres, nerve conduction velocity significantly decreased in rats with preceding treatment with cisplatin compared to those treated with the solvent for cisplatin.

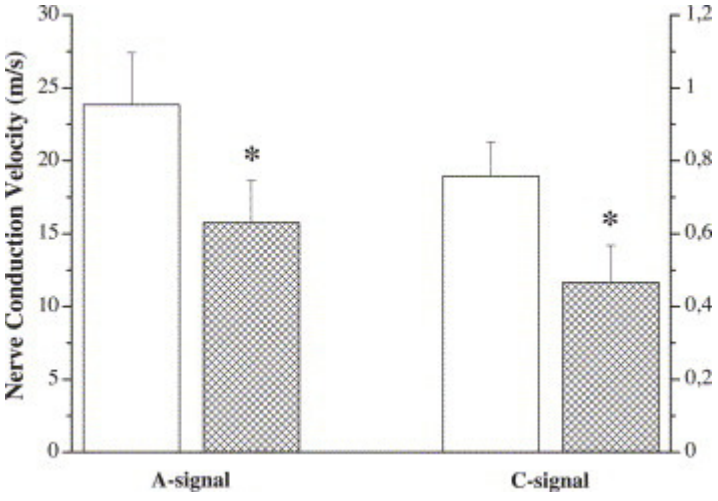

Fig. 2. Cisplatin-induced decrease in fast conducting myelinated (left ordinate) and slow conducting unmyelinated (right ordinate) fibres of the femoral nerve. Measurements were done 11 days after a series of five intraperitoneal injections of cisplatin (1.5 mg/kg/day) and/or its vehicle. The data are means±S.D. obtained from eight animals per group. *Significantly different from control at P≤0.05.

21.2.5.4 Changes in isometric tension

Preparations from normal animals exhibited a biphasic response to electrical field stimulation i.e. an initial contraction was followed by relaxation (Fig. 3). The rings from cisplatin-treated

rats responded with attenuated contractions to field stimulation compared to those from solvent-treated animals (Fig. 3). Nevertheless, the relaxation response to the field-stimulation protocol applied was of higher amplitude and shorter duration in rings from the cisplatin-treated animals than in controls. Field stimulation failed to induce any change in tension in rings pre-incubated with tetrodotoxin (data not shown).

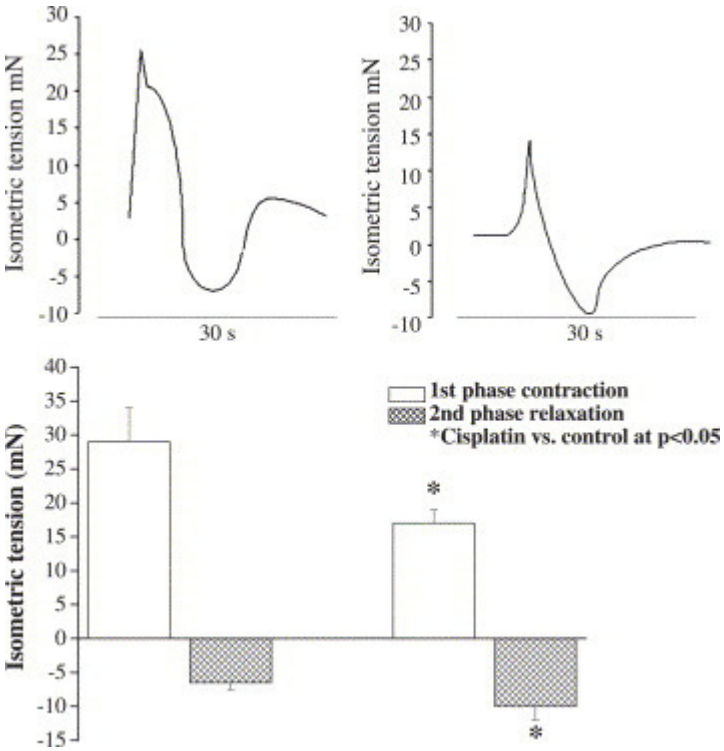

Fig. 3. Original tracings (top) and summarizing data (bottom) representing changes in isometric tension in bronchial preparations from control (left tracing and columns) and cisplatin-treated (right) animals in response to electrical field stimulation. Note the decrease in contractions in preparations from the cisplatin-treated animals. The data are means±S.D. obtained from eight values per group. *Significantly different from control at $P \leq 0.05$.

21.2.5.5 Sensory neuropeptide release

It is seen from data in Fig. 4 that the field stimulation-induced increase in sensory neuropeptide release was significantly attenuated in preparations from animals with sensory neuropathy induced by cisplatin.

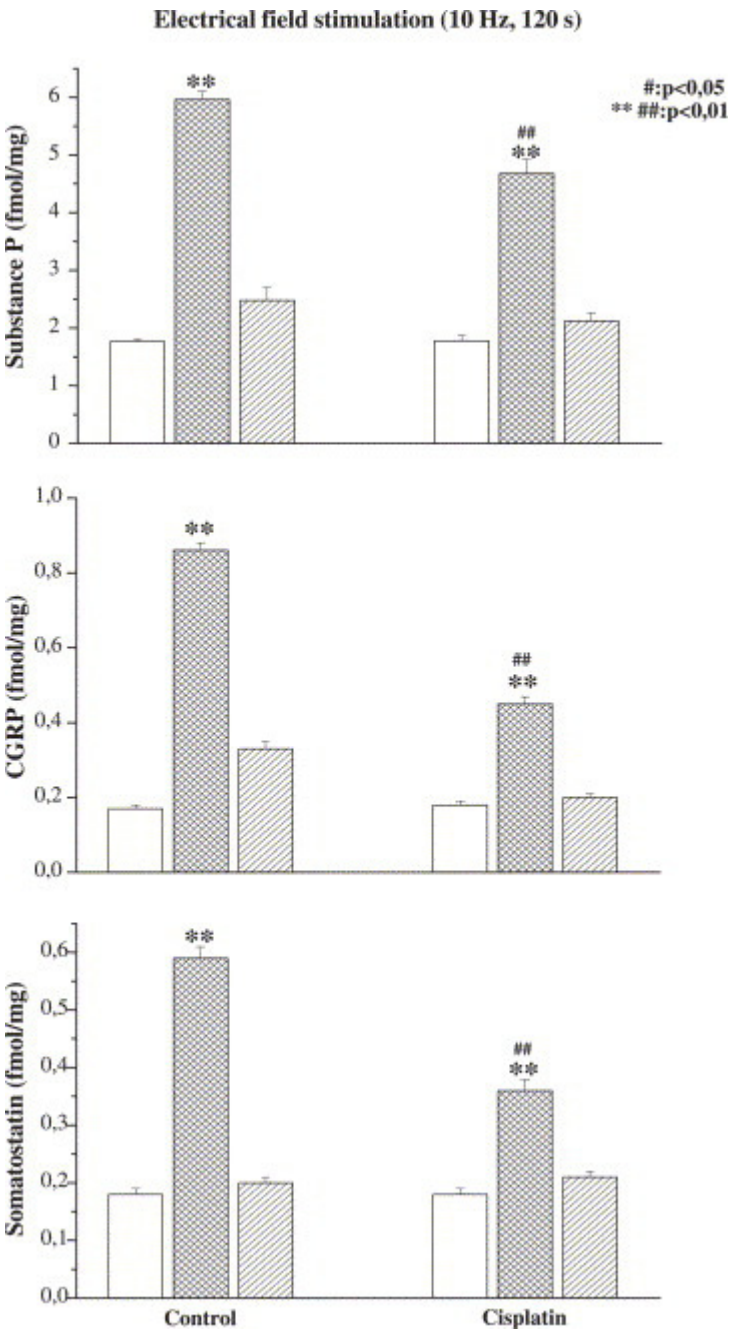

Fig. 4. Effect of field stimulation on sensory neuropeptide release from bronchial preparations from normal (vehicle-treated), and cisplatin-treated rats. Determinations were done prior to (open bars) as well as immediately (cross-hatched bars) and 2 min after (hatched bars) field stimulation The data are means±S.D. obtained from eight preparations per group. *Significantly different from corresponding 'normal' values at $P<0.05$.

21.2.6 *Discussion*

These results confirm previous findings obtained in guinea-pigs (Szilvassy et al., 2000) that a 5-day treatment with cisplatin attenuates field stimulation-induced bronchoconstriction in vitro in preparations from rats. This attenuated bronchomotor response occurs in parallel with a significant decrease in the release of sensory neuropeptides known to play a role in neurogenic regulation of bronchomotility such as that of somatostatin, substance P and CGRP in response to a highly standardized field stimulation challenge. This parallelism is the major

original finding of the paper. The field stimulation-induced bronchomotor response and the neuropeptide release were blocked by tetrodotoxin, a fast Na^+ channel blocker, thus, both can be considered to be of neural origin. These decreased responses were accompanied by a decline of femoral nerve conduction velocity in the cisplatin-treated animals. Since the nerve conduction velocity test is widely accepted as the 'gold standard' of peripheral neuropathy (Kato et al., 1998, Cameron and Cotter, 1997 and Love et al., 1996), it is also confirmed that the cisplatin-treated animals suffered from sensory neuropathy 11 days after the 5-day treatment period.

Bronchial tissue is densely innervated by unmyelinated sensory fibres containing substance P, CGRP, somatostatin and neurokinin A (Lundberg et al., 1983 and Lundberg et al., 1984). These fibres originate from the vagus nerve with cell bodies in jugular, nodose and dorsal root ganglia (Springall et al., 1987). As far as the regulatory role of these fibres in bronchomotility is concerned, it is closely linked to the so-called sensory effector function of these fibres. The essence of this particular function is that these fibres release their neurotransmitters into adjacent areas subsequent to activation attained by various stimuli such as an increase in extracellular K^+ concentration, decrease in pH (tissue acidosis) or electrical stimulation either with or without involvement of local reflexes (Szolcsanyi, 1996, Szolcsanyi et al., 1998 and Németh et al., 2003). The neurotransmitters, once released, produce various responses, for example, in case of CGRP and substance P, changes in vascular tone and permeability and/or bronchoconstriction (Lundberg et al., 1983 and Lundberg et al., 1984). As a methodological approach, these sensory nerve terminals locate in bronchial mucosa superficially enough to release neurotransmitters in response to electrical field stimulation at parameters selective for neural elements in sufficient quantities both to be detectable by analytical methods and to induce marked, predominantly NANC bronchoconstrictory responses. This enabled our experimental paradigm of studying neurogenic bronchomotiliy in relation to sensory neuropeptide release in cisplatin neuropathy in vitro. Since sensory neuropeptides play a major modulatory role in NANC bronchial motility, previous studies anticipated that a decreased availability of excitatory neuropeptides to be released by field stimulation were responsible for the feeble NANC contractile responses in bronchial preparations from cisplatin-treated animals similar to sensory neuropathy associated with advanced diabetes (Nemeth et al., 1999b and Szilvassy et al., 2002).

Of course, the current work can not be aimed to overemphasize the role of sensory neuropeptides in bronchomotility by that the experiments were focused on neuropeptide release. Our previous work revealed that it was the slow component of the bronchial contractile response to electrical field stimulation (shown to be capsaicin-sensitive), which disappeared in cisplatin neuropathy, whereas only a moderate decrease in the fast component (shown to be atropine- sensitive) was seen in preparations from such animals (Szilvassy et al., 2000). There is no doubt that acetylcholine is a neurotransmitter of crucial importance in bronchoconstrictive responses to electrical stimulation in both rats and several other species. As shown in Fig. 3, some of the rapid component of the bronchial contractile pattern is preserved in our cisplatin neuropathy model, which suggests that cholinergic pathways are also connected to some degree. The contractile pattern in Fig. 3 discloses the complete lack of the slow component of the field stimulation-induced contraction in preparations from cisplatin-treated animals, which is in good correlation with the significant decrease in the release of the three sensory neuropeptides measured.

The cisplatin treatment schedule applied yielded characteristic features of sensory neuropathy in that a significant decrease in femoral nerve conduction velocity occurred in both fast and

slow conducting fibres. This is in good accordance with our previous results in which a similar subacute treatment with high dose cisplatin induced peripheral neuropathy in guinea pigs (Szilvassy et al., 2000). Regarding the mechanism underlying cisplatin-induced peripheral neuropathy, it has been found that the primary targets for cisplatin neurotoxicity are the dorsal root ganglia cells (Gispen et al., 1992 and Barajon et al., 1996). In these cells, cisplatin induces an accumulation of sensory neuropeptides, suggesting an impaired axonal transport of sensory neurotransmitters to peripheral axon terminals (Barajon et al., 1996). Since the effector function of bronchial sensory nerves is underpinned by sensory neuropeptide release, it is not surprising that cisplatin-induced peripheral sensory neuropathy is reflected in impaired neurogenic bronchoconstriction as a consequence of a decrease in peripheral neuropeptide availability.

Theoretically, another possibility is that the enhanced relaxation response of preparations from cisplatin-treated animals to field stimulation may mask the entire contractile response showing contractions smaller than those seen in control. As shown in one of our previous papers, the exaggerated tracheal relaxation response associated with cisplatin neuropathy is underlain by an increased superoxide production (Szilvassy et al., 2000), at least in preparations from guinea pigs. Since this relaxation could completely be blocked by pharmacological inhibition of NO synthesis, it was concluded that this might have been related to an increase in peroxinitrite production, which otherwise is characteristic of cisplatin-induced neuropathy (Matsushima et al., 1998).

To the best of our knowledge, this work is the first to describe that the attenuated bronchomotor response in cisplatin-induced sensory neuropathy is related to a decrease in sensory neuropeptide release. Since non-adrenergic, non-cholinergic contractile agents such as substance P and CGRP together with tachykinins play an important role in neurogenic bronchoconstriction (Lundberg and Lou, 1996 and Szolcsanyi, 1996), this means that bronchi of animals suffering from peripheral neuropathy characterized by a decreased availability of sensory neuropeptides are less prone to contract in response to neural and antigen challenges. Besides diabetes (Szilvassy et al., 2002), cisplatin-induced neuropathy is another example for this phenomenon. Beyond providing some approach as to why bronchial hyper-reactivity is attenuated in diabetes and drug-induced peripheral sensory neuropathy, the results also call attention to pharmacological exploitation of the sensory neuropeptide release/effect-bronchial smooth muscle contraction pathway to confer protection on patients at risk of bronchial hyper-reactivity.

21.2.7 *Acknowledgements*

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21.3 Changes in tracheo-bronchial sensory neuropeptide receptor gene expression pattern in rats with cisplatin-induced sensory neuropathy

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21.3.1 *Abstract*

An attenuated neurogenic broncho-constriction underpinned by a decrease in sensory neuropeptide release has been shown to be characteristic of cisplatin-induced neuropathy. The present work was to explore if beyond neuropeptide release, cisplatin at a treatment schedule attaining sensory neuropathy, produced changes in the expression of the receptors of sensory neuropeptides such as somatostatin, calcitonin gene-related peptide (CGRP) and substance **P** (SP) in bronchial tissue of the rat. Twenty-four Wistar rats were divided into three groups. The animals in the "Treatment groups 1 and 2" were given cisplatin (1.5 mg kg^{-1}) and mannitol (75 mg kg^{-1}) over 5 days. The rats in the "Control" group were given mannitol + isotonic saline. Four animals from each group were used to study the expression pattern of the neuropeptide receptors in bronchial tissue. The levels of somatostatin receptor 4 (SSTR 4), neurokinin 1 (NK1), neurokinin 2 (NK2) and CGRP receptor expression were examined by quantitative real time polymerase chain reaction (RT-PCR) method, 11 and 22 days after the last cisplatin/vehicle dose. The cisplatin treatment significantly increased plasma somatostatin immunoreactivity and the expression of SSTR4 receptor detected both on the 11th and 22nd post-treatment days with no change in either CGRP, NK1, and NK2 receptor gene expression or plasma CGRP and substance **P** levels. We conclude that cisplatin neuropathy is accompanied by an increase in plasma somatostatin immunoreactivity with an increase in SSTR4 expression in rats.

Keywords: QRT-PCR; Somatostatin; Calcitonin gene-related peptide; Substance P; Radioimmunoassay; Cisplatin; Neuropeptide receptors; Neuropathy

21.3.2 *Article Outline*

21.3.3 *Introduction*

Cisplatin (diamino-dichloro-platinum) is chemotherapeutic agent widely used for the treatment of various types of cancer (Bardos et al., 2003). However, the therapeutic potential of the drug is strongly limited by its diverse side effects such as myelo-suppression, ototoxicity, nephrotoxicity and neurotoxicity. As far as the latter is concerned, cisplatin-induced neuropathy is predominantly of peripheral sensory nature characterized by a significant impairment of the effector function of vagal afferent sensory fibers innervated the bronchial tree (Szilvassy et al., 2000 and Horvath et al., 2005). This was shown to be underpinned by a decreased neuropeptide release, namely, a significant decrease in the release of CGRP, substance P and somatostatin were seen in response to electrical field stimulation in bronchial preparations from animals, who had been made neuropathy by means of a preceding period of cisplatin treatment (Horvath et al., 2005). The decreased neuropeptide release seemed to be physiologically relevant as it paralleled a strong attenuation in broncho-constrictive responses to electrical field stimulation in both guinea pigs and rats (Szilvassy et al., 2000 and Horvath et al., 2005). Since the decrease in transmission classically changes particular neurotransmitter receptors expression, the present work was concerned with the possibility that sensory neuropeptide receptor expression may change in cisplatin neuropathy (Baluk et al., 1997).

21.3.4 *Methods*

21.3.4.1 Ethics

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of Universities of Pécs and Debrecen, Hungary.

21.3.4.2 Experimental groups and animals

Fifty-two Wistar rats weighing 300–350 g were used throughout the experiment. Twenty-four animals selected for the QRT-PCR and RIA studies were randomized into two experimental groups. Control: animals treated with the solvent for cisplatin, 1 ml isotonic NaCl with 75 mg kg^{-1} mannitol, i.p., once a day over 5 days. The animals in the “cisplatin-treated” group were given 1.5 mg kg^{-1} cisplatin with 75 mg kg^{-1} mannitol, i.p., once a day over 5 days (Bardos et al., 2003). Four animals of the Control and Cisplatin group were used for studying

of the expression pattern of the neuropeptide receptors. Total mRNA was isolated 11 and 22 days following cisplatin treatment. Thirty-six animals were randomized for the measurement of the nerve conduction velocity studies.

21.3.4.3 Nerve conduction velocity

This series of experiments was carried out to verify/exclude sensory neuropathy involving unmyelinated slow conducting 'C'-fibers previously shown to play an important role in the HISS mechanism. Left saphenous nerve conduction velocity was determined in animals from both groups as described (Szilvassy et al., 2000, Nemeth et al., 1999a and Nemeth et al., 1999b). Briefly, in thiopentone-anaesthetized animals the nerve was prepared, cleaned of fat and adhering connective tissue and trains of square-wave constant voltage stimuli were applied through a pair of platinum electrodes (Experimetria Ltd., UK) placed as high as possible. The intensity, frequency and number of stimuli varied but the pulse width (500 μs) was kept constant. Another pair of electrodes was applied approximately 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding 'A' and 'C' waves were determined, reflecting activation of populations of A- and C-fibers, respectively. Average conduction velocity (m s^{-1}) was calculated by dividing the distance between the stimulating and receiving electrodes by the interval between the end of the stimulatory impulses 20 stimuli and the appearance of the corresponding 'A' and 'C' signals (Janig and Lisney, 1989).

21.3.4.4 Radioimmunoassay and blood glucose level

Plasma levels of CGRP, substance P (SP), insulin and somatostatin (SST) were measured by radioimmunoassay (RIA) developed in our laboratory (Nemeth et al., 1996, Nemeth et al., 1998 and Nemeth et al., 1999c). Detection limit for the SST and SP were 1 fmol ml^{-1} and for CGRP 0.1 fmol ml^{-1}, respectively. Blood glucose level was determined by means of the glucose oxidase method (Accu-Chek, Roche Diagnostics, Hungary). Peripheral insulin sensitivity and β-cell function was also determined using homeostasis model assessment (HOMA-IR = (FPI) × (FPG)/22.5 and HOMA-%B = 20 × FPI/FPG-3.5) in fasted animals (16 h period of fasting preceding sampling) as previously described (Matthews et al., 1985).

21.3.4.5 RNA isolation

The excised trachea and the main bronchi were immediately placed in RNA Later buffer (Qiagen, Inc.) and kept at −70 °C till processing. Total RNA was isolated with the RNA isolation kit according to the manufacturer protocol. The quality of the prepared RNA was determined by electrophoresis on agarose gel and the quantity was measured by ND1000 (Nanodrop, Inc.) spectrophotometer. Two hundred nanograms total RNA was used for each single quantitative RT-PCR assay.

21.3.4.6 Quantitative real time polymerase chain reaction

Quantitative real time polymerase chain reaction (QRT-PCR) was performed by the SybrGreen detection method. Primers were designed by the Primer 3 online program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). All primers were checked by BLAST, to reduce the possibility of the amplification of other genes. The primers were:

NK1 forward: tgggcaacgtagtggtgata, reverse: cacggctgtcatggagtaga;

NK2 forward: ggagagtcaaccggtgtcat, reverse: ccgagcaccattctgttttt;

CGRP receptor forward: agaacttgaacgccatcacc, reverse: ggatctcaacagcggtcatt;

SSTR4 forward: gccactgtcaaccatgtgtc, reverse: tcttcctcagcacctccagt;

Beta 2 microglobulin forward: acttcctcaactgctacg, reverse: tggtgtgctcattgctat.

21.3.4.7 Relative quantification of the examined genes

Beta 2 microglobulin (b2m) gene was used as internal control. Only those reactions were included in the quantitative analysis, which gave a well-defined amplification product both by melting curve analysis and agarose gel electrophoresis. To compare the different mRNA transcription levels, C_T values were compared directly. C_T is defined as the number of cycles needed for the fluorescence signal to reach a specific threshold level of detection, and is inversely correlated with the amount of specific template nucleic acid present in the reaction.

We compared the RNA transcription of the examined neuropeptide receptor genes with b2m. ΔC_T was first calculated between the C_T values at the 16th and 22nd day from samples from cisplatin-treated and control animals. In the second step, we subtracted the changes in RNA transcription in samples from control animals from the changes in samples from cisplatin-treated animals to obtain the $\Delta\Delta C_T$. This indicated changes in RNA transcription caused by cisplatin treatment between the 16th and 22nd day normalized to RNA transcription changes in the control samples. A high $\Delta\Delta C_T$ value, if negative or positive, indicated significant changes in the RNA transcription level of the tested gene. A positive $\Delta\Delta C_T$ value indicated down-regulation of RNA transcription, whereas a negative $\Delta\Delta C_T$ indicated an up-regulation of the gene's transcription following cisplatin treatment (Radonic et al., 2004).

$$\begin{aligned}\Delta\Delta C_{T11\ day} &= (C_{Receptor} - C_{b2m})_{11\ day} - (C_{Receptor} - C_{b2m})_{control}\\ \Delta\Delta C_{T22\ day} &= (C_{Receptor} - C_{b2m})_{22\ day} - (C_{Receptor} - C_{b2m})_{control}\end{aligned} \quad (1)$$

For normalizing the given data we used the $2^{-\Delta\Delta C_T}$ method (Bernard and Wittwer, 2002).

21.3.4.8 Study design

Fig. 1 shows the schematic diagram of the experimental protocol. The animals were treated with cisplatin over 5 days to induce sensory neuropathy. The animals treated with solvent for cisplatin over 5 days served as the control group. The animals from either group were divided into two subgroups; one for QRT-PCR and RIA measurements and another one for nerve conduction velocity determination. Each animal was fasted overnight before blood sampling

for plasma CGRP, substance P, somatostatin and insulin as well as fasting blood glucose. These determinations were done 11 and 22 days after cessation of the cisplatin treatment schedule.

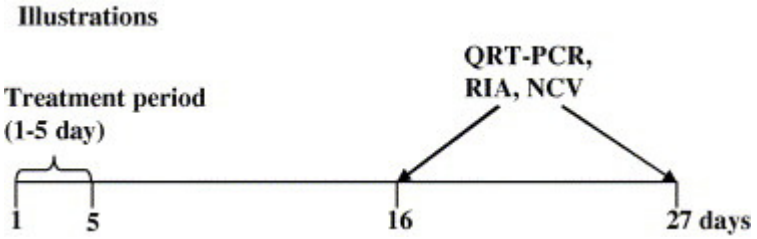

Fig. 1. The schematic representation of the experimental protocol. After the animals were treated with either cisplatin or its solvent over 5 days (1–5 day), QRT-PCR, RIA and nerve conduction velocity (NCV) were determined both on the 16th and 27th days.

21.3.4.9 Exclusion

Only those samples (four from each group) were used for QRT-PCR measurements, where the QRT-PCR analysis of the samples gave a well-defined amplification product both by melting curve analysis and agarose gel electrophoresis.

21.3.4.10 Drugs and chemicals

The chosen primers designed by using the Primer 3 online program (see section 2.6) were ultimately purchased from Sigma-Aldrich (Budapest, Hungary) Sigma-Aldrich, RNeasy Protect Mini Kit from Qiagen Inc. Quantitative Real Time Polymerase Chain Reaction (KasztelMed, Hungary Budapest) was carried out by LightCycler PCR machine (Roche Applied Science). For quantifying mRNA LightCycler RNA Master SYBR Green I kit was used from Roche Applied Science (KasztelMed, Hungary Budapest). Cisplatin was obtained from TEVA-BIOGAL, Debrecen. Chemicals used for radioimmunoassay determinations, were as follows: Tyr-α-CGRP was purchased from Bachem, Germany CGRP, substance P, somatostatin antiserum were a present of Dr. T. Görcs from the University Medical School of Budapest, Hungary. Polypropylene RIA tubes were obtained from Merck (Darmstadt, Germany). ^{125}I-labelled tracers were prepared in our laboratory (Nemeth et al., 2002).

21.3.4.11 Data analysis

Data of RT-PCR measurements are expressed by means of ± standard error of the mean (SEM). Changes were significant if the expression level differed more than two times from corresponding control values. The data obtained from RIA measurements are expressed as means of ± SEM and analyzed by ANOVA followed by Student's *t*-test supplemented with appropriate post hoc evaluation. In case of data normalization failure, the possibilities for further statistics were left by using Man–Whitney's *U*-test. Changes considered significant at $p \leq 0.05$.

21.3.5 *Results*

21.3.5.1 Nerve conduction velocity

Table 1 shows that cisplatin, at the treatment schedule applied, produced a significant decrease in nerve conduction velocity in both fast conducting myelinated 'A' and slow conducting unmyelinated 'C'-fibers determined either 11 or 22 days following cisplatin treatment (Table 1).

The effect of cisplatin treatment (1.5 mg kg^{-1}) on the nerve conduction velocity (NCV) of the myelinated Aδ and unmyelinated C-fibers

	NCV in A-fibers ($m^{-1}s^{-1}$)	**NCV in C-fibers ($m^{-1}s^{-1}$)**
Control	23.8 ± 3.5	0.77 ± 0.1
11 day after treatment	15.8 ± 2.9*	0.48 ± 0.1*
22 day after treatment	17.4 ± 3.2*	0.53 ± 0.2*

Table 1: * Significant ($p \leq 0.05$) differences of the corresponding control value.

21.3.5.2 Plasma somatostatin, CGRP and substance P levels

It is seen in Fig. 2, that plasma somatostatin immunoreactivity significantly increased 11 and 22 days after cisplatin treatment. Plasma CGRP level exhibited only a transient increase, whereas plasma substance P did not reveal any change.

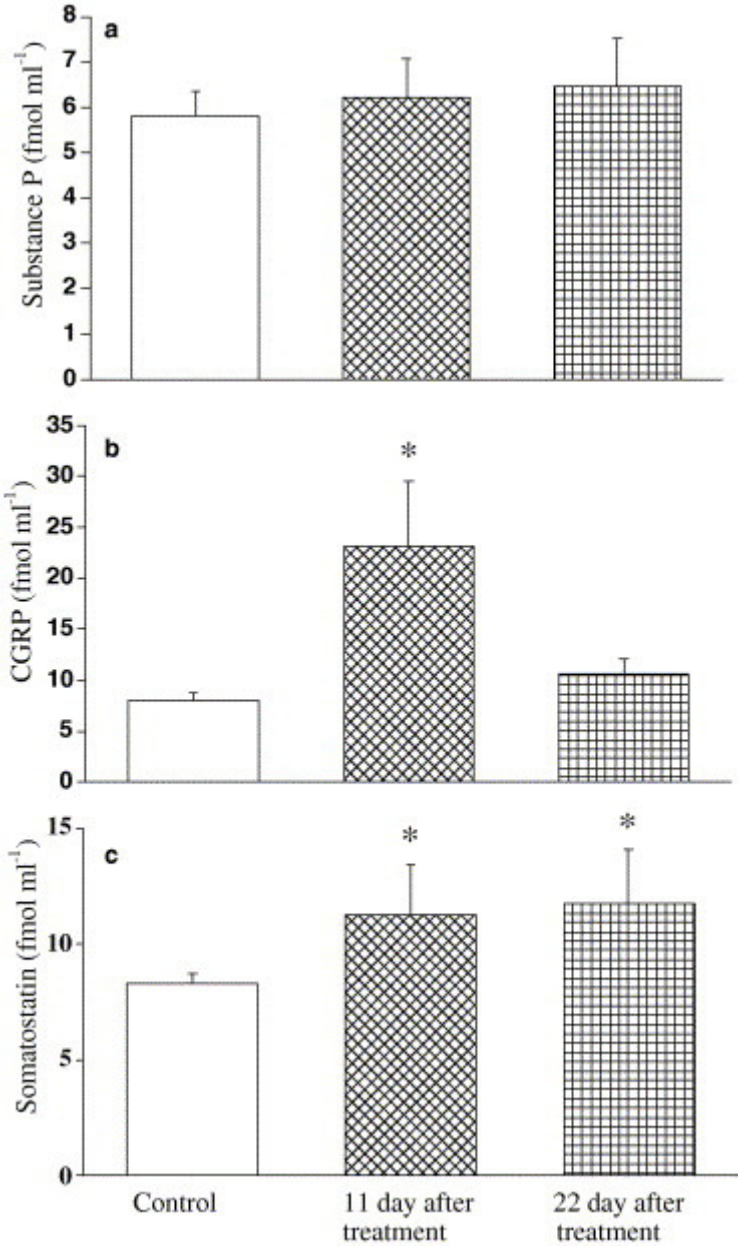

Fig. 2. Effect of cisplatin treatment on plasma substance P (a), CGRP (b) and somatostatin (c) levels (n = 8). Results are means ± SEM. *p ≤ 0.05 vs. control (mannitol + isotonic saline).

21.3.5.3 Plasma insulin and glucose level

Table 2 shows the effect of 5 day solvent/drug administration on plasma insulin and glucose levels in control and cisplatin-treated rats. Both plasma insulin and fasting blood glucose levels remained unaffected by cisplatin treatment. In accordance with these results, the HOMA-IR and HOMA-%B did not show any significant difference between the control and Cisplatin-treated group (Table 2).

	Solvent-treated group		Cisplatin-treated group	
	16th day	**27th day**	**16th day**	**27th day**
Plasma insulin (μU ml^{-1})	9.4 ± 1.4	9.5 ± 0.5	9.6 ± 1.2	9.5 ± 0.4
Plasma glucose (mmol l^{-1})	4.5 ± 0.1	5.1 ± 0.2	5.7 ± 1.0	6.0 ± 1.0
HOMA-IR	1.9 ± 0.1	2.1 ± 0.0	2.4 ± 0.1	2.5 ± 0.1
HOMA-%B	180.4 ± 19.4	120.6 ± 14.3	88.3 ± 10.1	75.8 ± 6.9

Table2 Shows that cisplatin neuropathy did not modify glucose homeostasis as determined by the measurement of the fasting plasma insulin and glucose levels and as expressed by HOMA-IR and HOMA-%B

21.3.5.4 RT-PCR measurements

On the 16th day of the experiment the expression of the NK1, NK2 and CGRP receptor mRNAs increased 3.22 ± 1.29, 2.78 ± 1.14 and 1.31 ± 0.14 times the control level, respectively. On the 27th day of the experiment the expression level of the NK1, NK2 and CGRP receptor mRNA decreased to 0.69 ± 0.28, 0.91 ± 0.24 and 0.62 ± 0.07 times to the control, respectively (i.e., the difference is non-significant as compared by the 0 day control values). On the other hand, the expression pattern of SSTR4 mRNA is completely different from the above mentioned neuropeptides receptors mRNA expression pattern. We found a significant increase not only at the 16th day, but also at the 27th day as well. The expression level of the mRNA of the SSTR4 increased 4.41 ± 2.48 times on the 16th day and 7.72 ± 2.66 times on the 27th day (Fig. 3).

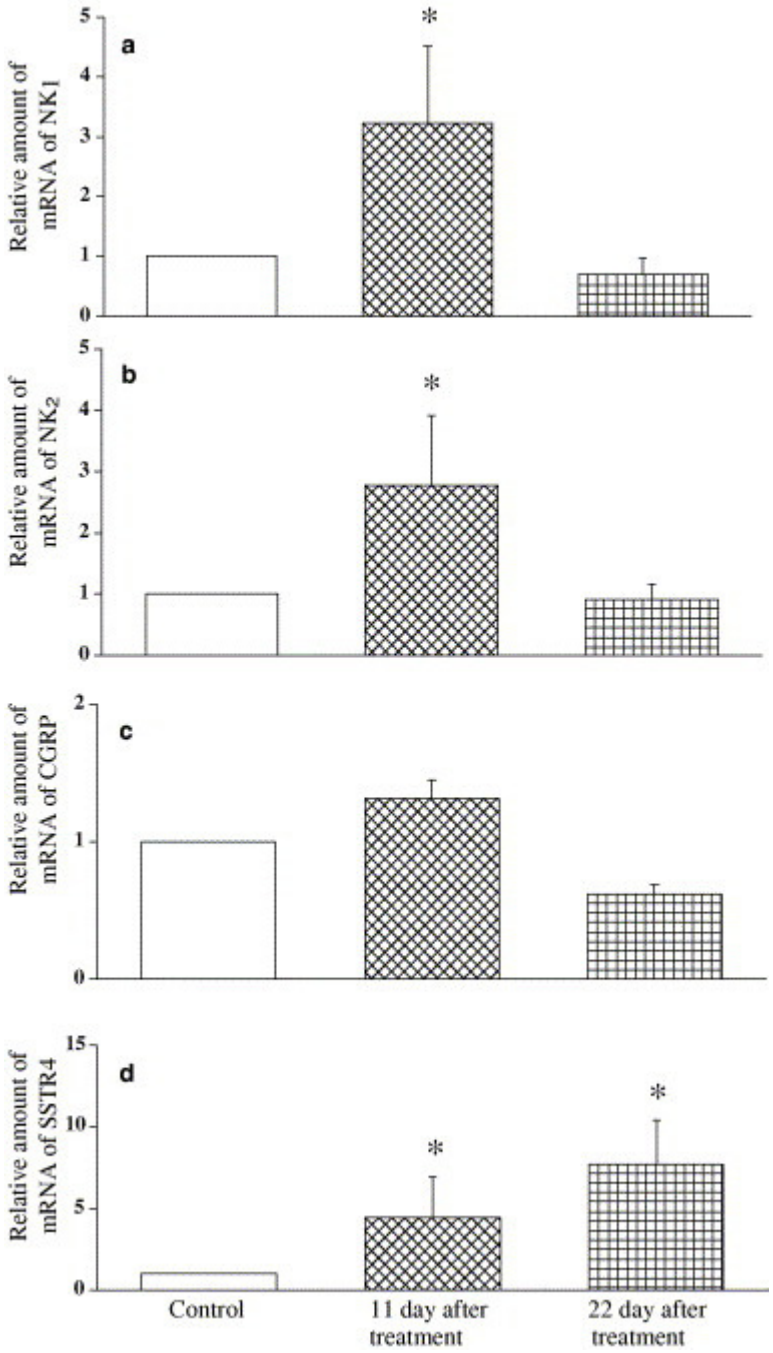

Fig. 3. Changes in the expression pattern of NK1 (a), NK2 (b), CGRP (c) and somatostatin (SSTR4) receptors (d) induced by cisplatin neuropathy ($n = 4$). The results are means ± SEM. '*' indicates that changes in receptor expression exceed at least two times that obtained in samples from animals treated with mannitol + isotonic saline (control).

21.3.6 *Discussion*

The animals treated with cisplatin exhibited sensory neuropathy characterized by a significant decrease in nerve conduction velocity in unmyelinated C-fibers. The treatment schedule applied has previously been shown to significantly decrease the release of sensory neuropeptides in response to electrical field stimulation in bronchial preparations from rats that has been suspected to underlie an attenuation of non-adrenergic, non-chlonergic broncho-constriction (Horvath et al., 2005). The major original finding of the current work, however, is

that cisplatin-induced sensory neuropathy beyond decreasing sensory neuropeptide release, induces selective over-expression of SSTR4 with no change in the expression of either CGRP or NK1 and NK2 receptors in rat bronchial tissue. Moreover, this occurred in parallel with a significant increase in plasma somatostatin immunoreactivity with no change in plasma CGRP and substance P levels.

Bronchial tissue is densely innervated by unmyelinated sensory fibers containing substance P, CGRP, somatostatin and neurokinin A (Lundberg et al., 1983 and Lundberg et al., 1984). These fibers originate from the vagus nerve with cell bodies in jugular, nodose and dorsal root ganglia (Springall et al., 1987). As far as the regulatory role of these fibers in broncho-motility is concerned, it is closely linked to the so-called sensory effector function of these fibers. The essence of this particular function is that these fibers release their neurotransmitters into adjacent areas subsequent to activation attained by various stimuli such as an increase in extracellular K^+ concentration, decrease in pH (tissue acidosis) or electrical stimulation either with or without involvement of local reflexes (Szolcsanyi, 1996, Szolcsanyi et al., 1998 and Nemeth et al., 2003). The neurotransmitters, once released, produce various responses, for example, in case of CGRP and substance P, changes in vascular tone and permeability and/or broncho-constriction (Lundberg et al., 1983 and Lundberg et al., 1984). As a methodological approach, these sensory nerve terminals locate in bronchial mucosa superficially enough to release neurotransmitters in response to electrical field stimulation at parameters selective for neural elements in sufficient quantities both to be detectable by analytical methods and to induce marked, predominantly NANC broncho-constrictory responses. This enabled our former experimental paradigm of studying neurogenic broncho-motiliy in relation to sensory neuropeptide release in cisplatin neuropathy in vitro (Szilvassy et al., 2000 and Horvath et al., 2005). Since sensory neuropeptides play a major modulatory role in NANC bronchial motility, previous studies anticipated that a decreased availability of excitatory neuropeptides to be released by field stimulation were responsible for the feeble NANC contractile responses in bronchial preparations from cisplatin-treated animals similar to sensory neuropathy associated with advanced diabetes (Nemeth et al., 1999b and Szilvassy et al., 2002). Interestingly, a decreased sensory neuropeptide release from bronchial tissue occurred in parallel with a significant increase in plasma somatostatin immunoreactivity, whereas plasma levels of the other sensory neuropeptides such as CGRP and substance P did not change in neuropathy produced by either diabetes or neurotoxic doses of cisplatin as revealed by the results of the present work (Szilvassy et al., 2002). As far as the explanation for the increased plasma level of somatostatin in our diabetes models is concerned, we postulated that it resulted from hyperglycemia, since the release mechanism of somatostatin from pancreatic delta cells regulated by ATP-sensitive potassium channels resembles that seen for insulin in beta cells (Nemeth et al., 1999b and Szilvassy et al., 2002). Nevertheless, cisplatin neuropathy did not modify blood glucose level; therefore the cisplatin neuropathy-induced relative hypersomatostatinaemia (elevated plasma levels of somatostatin) should be attributed to other mechanisms.

The most striking original finding of the present work, however, is that hypersomatostatinaemia seen in animals with cisplatin-induced neuropathy is accompanied by an increased expression of SST4 receptors, at least in bronchial tissue. These receptors have been shown to mediate anti-inflammatory and analgesic effects underpinned by a suppression of neuropeptide release with no influence on insulin secretion (Helyes et al., 2001, Pinter et al., 2002 and Olias et al., 2004). Taken the increased SSTR4 expression together with hypersomatostatinaemia it is strongly suggested that cisplatin might be of particular

importance as a therapeutic tool in patients with painful endocrine tumors.

21.3.7 *Acknowledgments*

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21.4 Feeble bronchomotor responses in diabetic rats in association with decreased sensory neuropeptide release

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21.4.1 *Abstract*

Type I diabetes is associated with a low incidence of asthma. We tested whether a decrease in sensory neuropeptide release is associated with an attenuated bronchoconstrictive response to field stimulation (FS; 100 stimuli, 20 V, 0.1 ms, 20 Hz) in streptozotocin (STZ)-induced diabetes. The organ fluid of the preparations were also tested for substance P, calcitonin gene-related peptide (CGRP), and somatostatin concentrations by RIA. Preparations were from either normal rats or those pretreated with 50 mg/kg STZ iv 8 wk before experiment. A group of STZ-treated animals was supplied with insulin delivery (4 IU/day sc) implants between 4 and 8 wk. A subgroup was formed to study the effect of capsaicin desensitization. The atropine-resistant contraction was attenuated by diabetes without capsaicin-sensitive relaxation response. Exogenous CGRP and substance P potentiated, whereas somatostatin inhibited (1 nM-10 μM) the FS-induced contractions in rings from either group. FS released somatostatin, CGRP, and substance P from 0.17 ± 0.024, 0.15 ± 0.022, and 1.65 ± 0.093 to 0.58 ± 0.032, 0.74 ± 0.122, and 5.34 ± 0.295 in preparations from normal, and from 0.19 ± 0.016, 0.11 ± 0.019, and 0.98 ± 0.116 to 0.22 ± 0.076, 0.34 ± 0.099, and 1.84 ± 0.316 fmol/mg wet wt in preparations from diabetic rats. Insulin supplementation restored neuropeptide release in rings from STZ-treated rats. The results show that the decreased FS-induced contractions occurred with a decrease in sensory neuropeptide release in STZ-diabetic rats. diabetes; bronchoconstriction; capsaicin

21.4.2 *Introduction*

TYPE I DIABETES IS ASSOCIATED with a low incidence of asthma (2, 7, 13). The proposal that epithelial damage determines bronchial hyperreactivity (1) presupposes a central role of vagal reflexes supported by a range of findings. Manifestations of hyperreactivity in vivo are not paralleled by altered sensitivity to spasmogens when strips or rings of isolated airway smooth muscle from hyperreactive animals are studied in vitro (33). Furthermore, some forms of hyperreactivity can be prevented by sectioning of the vagus nerve (26). Considering that almost 90% of the vagal nerve comprises sensory fibers, it is not surprising that capsaicin pretreatment can prevent some forms of airway hyperreactivity (22). Alternatively, infusion of sensory neuropeptides induces hyperreactivity in guinea pigs (8). We have found that the release of sensory neuropeptides, such as that of calcitonin gene-related peptide (CGRP), substance P, and somatostatin, is significantly decreased from isolated tracheae of rats with diabetic sensory neuropathy (20). Given this decrease in sensory neuropeptide release together with the well-documented attenuation of contractile responses of tracheal preparations from insulin-deficient rats to field stimulation (FS) in other studies, we sought to find whether there would be an association between the two processes in the same set of experiments. Here we show that attenuation of field stimulation-induced contractions of the bronchial rings from diabetic rats closely relates to a deficient sensory neuropeptide release.

21.4.3 *Methods*

21.4.3.1 Ethics.

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical boards of the Medical Universities of Pécs and Debrecen, Hungary.

21.4.3.2 Experimental groups

The study was carried out with 48 male Wistar rats weighing 200-210 g and 12 male Dunkin-Hartley guinea pigs (400-420 g). They were housed in an animal room (12-h light/dark periods a day, temperature of 22-25°C, humidity of 50-70%) with four animals per pen and fed commercial laboratory chow and tap water ad libitum. The animals were randomly divided into two experimental groups. The control animals were treated with the solvent for streptozotocin (STZ), whereas the rats in the second group were treated with 50 mg/kg STZ iv (Zanosar; Upjohn, Kalamazoo, MI) to make them diabetic. After 4 wk, the STZ-treated animals were further randomized into two additional groups, one of which comprised animals that were supplied with continuous-delivery (4 IU/day) subcutaneous insulin implants (Linplant; Mollegaard, Ejby, Denmark). This group was referred to as the insulin-supplemented group. The implants were placed at the back of the neck under Trapanal anesthesia (45 mg/kg ip). The other subgroup of rats received matching placebo implants (diabetic group). From the diabetic and the insulin-supplemented groups of animals, we formed subgroups to study the effect of pretreatment with neurotoxic capsaicin doses on FS-induced bronchomotor responses and neurotransmitter release studies (n = 6 animals per subgroups)

21.4.3.3 Isometric tension measurements.

Isolated segments of the main bronchi (2 mm) were mounted horizontally on two small L-shaped glass hooks, one of which was connected to a force transducer for measurement of isometric tension. The experiments were carried out in thermostatically controlled (37 ± 0.2°C) organ bath (5 ml) (TSZ 02, Experimetria UK, London, UK) containing Krebs solution. The organ fluid was gassed with 95% O_2 and 5% CO_2 to maintain pH at 7.40 ± 0.05. Neural effects on contractile activity of the segments were studied by means of FS (100 stimuli at 20 V, 0.1 ms, and 20 Hz at an initial tension of 12 mN). The rings were prepared from six animals in each group. To study whether the FS protocol applied was selective for nerve-mediated responses, we preincubated some rings for a period of 10 min with tetrodotoxin (TTX), a fast sodium channel blocker.

21.4.3.4 Neurotransmitter release studies.

These have been described in detail elsewhere (20). In brief, after the animals were killed by exsanguination, the lower third of the tracheae with the main bronchi were removed and cleaned of fat and adhering connective tissues. They were prepared for perfusion in a temperature (37°C)- and pH (7.2)-controlled, oxygenized Krebs solution over 60 min. Electrical FS (40 V, 0.1 ms, 10 Hz for 120 s) was applied to elicit neurotransmitter release. CGRP, substance P, and somatostatin concentrations were determined from 200-μl samples of organ fluid of the preparations by means of RIA methods developed in our laboratories as described previously (20).

21.4.3.5 Determination of plasma somatostatin, insulin, and blood glucose concentrations.

Arterial blood samples (3 ml/rat) were taken into ice-cold tubes containing EDTA (6 mg) and Trasylol (1,000 IU). The samples were then centrifuged at 4°C (2,000 rpm for 10 min). The somatostatin content of 1 ml plasma was extracted by addition of three volumes of absolute ethanol. After precipitation and a second centrifugation with the same parameters, the supernatants were aspirated and subsequently evaporated under nitrogen as described (9). Plasma somatostatin immunoreactivity was determined by means of RIA (19, 30, 31). Plasma insulin and blood glucose levels were measured by RIA and the glucose peroxidase method, respectively.

21.4.3.6 Nerve conduction velocity studies.

This series of experiments was carried out to verify or exclude diabetic sensory neuropathy. Left saphenous nerve conduction velocity was determined in subgroups of normal, diabetic, and STZ plus insulin-treated animals. In artificially ventilated animals anesthetized with thiopental sodium (50 mg/kg ip), the nerve was prepared and cleaned of fat and adhering connective tissues, and strains of square-wave (500 μs) constant-voltage stimuli were applied through pairs of platinum electrodes placed as high as possible. Another pair of electrodes was applied 2 cm distal to the stimulating electrodes for recording the summation action potentials evoked by the proximal stimulation. The time lags between stimulation and the appearance of corresponding A and C signals were determined for calculation of average conduction velocity by dividing the interelectrode distance by the interval between the end of the stimulatory impulse and the appearance of the A and C signals (14).

21.4.3.7 Treatment with capsaicin.

Capsaicin was used to elicit a selective functional deterioration of a significant portion of sensory C fibers. Rats constituting subgroups from normal and diabetic animals (n = 6 for each) were given capsaicin/solvent subcutaneously in the sequence of 10, 30, and 50 mg/kg single daily doses over 3 days on the 8th wk of the experimental period. Capsaicin (1% wt/vol) was dissolved in physiological saline containing 3% vol/vol ethanol and 4% vol/vol Tween 80. The animals pretreated with capsaicin were used for further studies after a 3-day period of recovery to avoid nonspecific effects of systemic capsaicin administration as described previously (10).

21.4.3.8 Mechanical responses to ovalbumin in isolated tracheae from sensitized normal and diabetic guinea pigs.

Twelve male Dunkin-Hartley guinea pigs (400-420 g) were randomized into two groups. The control animals were treated with the solvent for STZ, whereas the second group of animals was treated with a single intraperitoneal injection of 180 mg/kg STZ (Zanosar). Four weeks after STZ and/or solvent injection, the animals were actively sensitized by two intraperitoneal injections of 1 ml/kg 5% (wt/vol) ovalbumin (grade III; Sigma, St. Louis, MO) on two consecutive days. The animals were killed after an additional period of 4 wk for isolated trachea experiments.
The trachea was cut into single rings that were tied together forming up to six four- to five-ring chains and suspended in 15-ml organ baths containing temperature (37°C)- and pH (7.2)-controlled Krebs buffer continuously aerated with carbogen. The ends of the chains were tied at the bottom of the tissue bath and connected to a force transducer for measurement of isometric tension (TSZ 03, Experimetria UK). The initial tension was set at 15 mN, and after an equilibration period of 60 min the chains were exposed to cumulative increases in ovalbumin concentration (10^{-11}-10^{-7} g/ml organ bath volume). When the maximum contraction to ovalbumin had been reached, 3 mM carbachol was additionally applied to define the maximum contraction of each tracheal chain. The contractile responses to ovalbumin were expressed as percentage values of the carbachol-induced maximum responses. Only one concentration-response curve of ovalbumin was generated with each chain.

21.4.3.9 Drugs and solutions.

Thiopental sodium (Trapanal, EGIS, Budapest, Hungary) was purchased from Byk Gulden (Konstanz, Germany), STZ (Zanosar) from Upjohn, guanethidine, atropine, somatostatin, CGRP, substance P, and TTX from Sigma, and capsaicin from Fluka (Buchs, Switzerland). Trasylol was from Richter (Budapest, Hungary), and insulin RIA kits were from Izinta (Budapest, Hungary). ^{125}I-labeled RIA tracers were prepared in our laboratory.

21.4.3.10 Experimental protocol.

Eight weeks after treatment with STZ or solvent, the animals were either exsanguinated for in vitro experiments and laboratory determinations or used for nerve conduction velocity studies. Food was withdrawn 12 h before blood sampling for glucose, plasma insulin, and somatostatin measurements. Insulin and somatostatin immunoreactivity were determined by means of RIA (19, 30, 31). The lower third of the tracheae with the main bronchi was then isolated for isometric tension measurements and neurotransmitter release studies. Six separate animals per group entered the nerve conduction velocity study group.

21.4.3.11 Statistical analysis.

The isometric tension and nerve conduction velocity data expressed as means ± SD were evaluated with analysis of variance followed by a modified *t*-test according to Bonferroni's method (32). The blood chemistry data and sensory neuropeptide levels were evaluated by Student's *t*-test for unpaired data.

21.4.4 *Results*

21.4.4.1 Effects of experimental diabetes on body weight, blood glucose, plasma insulin, and somatostatin levels.

The normal animals grew steadily over the 8-wk observation period with an average weight gain of 62 ± 4.1 and 58 ± 6.1 g, respectively. The diabetic animals exhibited a marginal weight loss (5.0 ± 2.1 g). The insulin-supplemented rats failed to grow during the first 4 wk. Insulin supplementation from the slow release implants (~4 IU/day) during *wk 4-8* caused a significant increase in body weight to a level approaching that seen in normal animals (Fig. 1). In normal, diabetic, and insulin-supplemented animals, fasting blood glucose levels were 4.4 ± 0.6, 17.4 ± 5.5, and 5.0 ± 0.6 mmol/l ($P < 0.001$ between diabetic vs. normal or insulin supplemented), with plasma insulin levels of 11.4 ± 3.2, 2.0 ± 0.4 ($P < 0.001$ vs. normal), and 12.9 ± 3.8 μIU/ml, respectively. Fasting plasma somatostatin level significantly increased in diabetic vs. normal animals. In response to insulin supplementation, plasma somatostatin level renormalized by the end of the 8-wk period (Fig. 2). Sampling for these determinations was done at the end of the 8-wk experimental period.

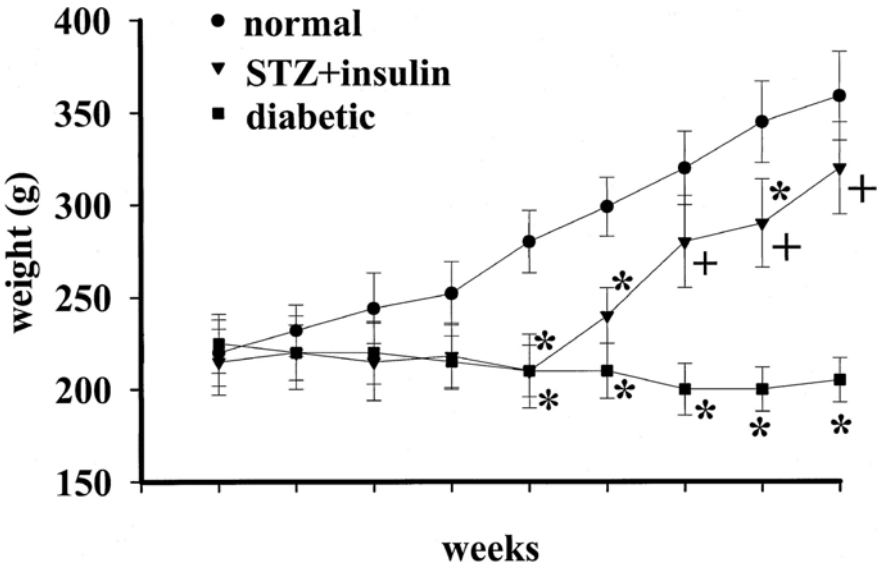

Fig. 1. Decrease in body weight by treatment with streptozotocin (STZ, 50 mg/kg iv) in rats. A reversal but not normalization by insulin. The rats were weighed weekly. The results are expressed as means ± SD obtained with 6 animals/group. Note that changes in body weight are based on data obtained with animals that did not receive treatment other than STZ, insulin, or solvents. For example, data obtained with animals treated with capsaicin are not included in the figure. The data are means ± SD obtained with 6 animals/group. * Different from normal at $P < 0.05$; +Difference between STZ+insulin and diabetic values at $P < 0.05$.

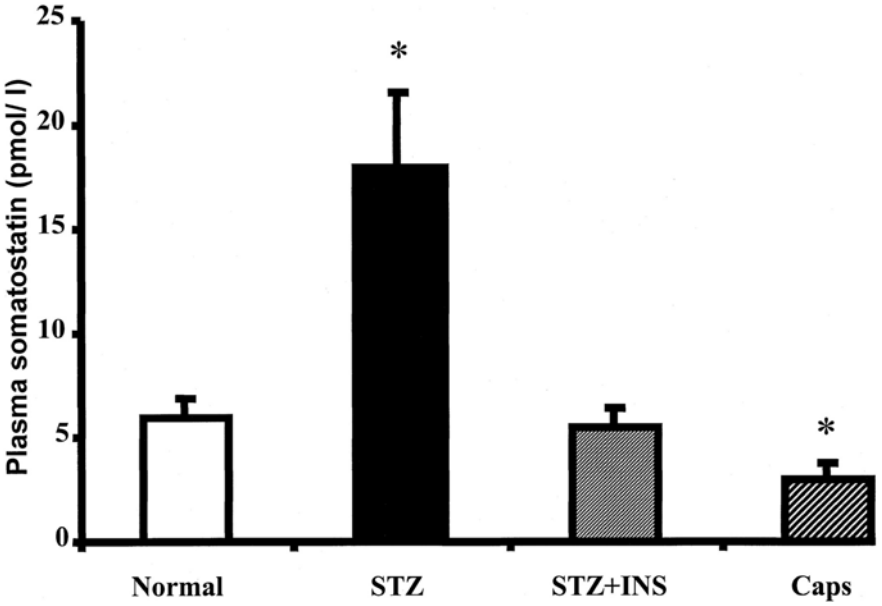

Fig. 2. Effect of treatment with STZ (50 mg/kg iv 8 wk before sampling) and capsaicin (Caps; sequential pretreatment) on plasma somatostatin levels as determined by means of RIA in rats. An interaction with insulin (INS, 4 IU/day started 4 wk after STZ and applied over 4 wk) supplementation. The data are means ± SD at $n = 6$ animals/group. * Significantly different from normal and STZ+insulin at $P < 0.05$.

The guinea pigs receiving the solvent for STZ exhibited a weight gain of 45 ± 6.4 g over the 8-wk observation period, whereas body weight of the STZ-treated animals did not show any change.

21.4.4.2 Nerve conduction velocity.

Figure 3 shows the diabetes-induced decrease in nerve conduction velocity in fast-conducting myelinated (A fibers in Fig. 3*A*) and slow-conducting unmyelinated (C fibers in Fig. 3*B*) fibers. At a stimulation intensity suprathreshold for A (0.5 V, 5 Hz) or C (3 V, 5 Hz) fibers, conduction velocity significantly decreased in diabetic rats. In the insulin-supplemented animals, conduction velocity for either A or C fibers did not differ from those determined in the control group.

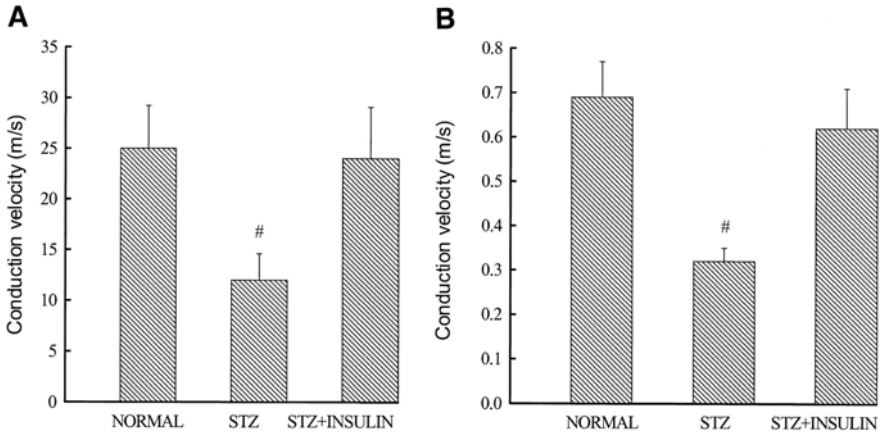

Fig. 3. Diabetes-induced decrease in nerve conduction velocity in fast-conducting myelinated (*A*) and slow-conducting unmyelinated (*B*) fibers of the femoral nerve. Measurements were accomplished 8 wk after a single 50 mg/kg iv dose of STZ. The data are means ± SD obtained with 6 animals/group. # Significantly different from normal and STZ+insulin at $P < 0.05$.

21.4.4.3 Contractile responses to FS.

Preparations from normal animals exhibited a biphasic response to FS, i.e., an initial contraction was followed by relaxation (Figs. 4 and 5*A*). The rings from diabetic rats responded with attenuated monophasic contractions to FS compared with those seen in preparations from normal or insulin-supplemented animals (Figs. 4 and 5*B*). FS failed to induce any change in tension in rings preincubated with TTX.

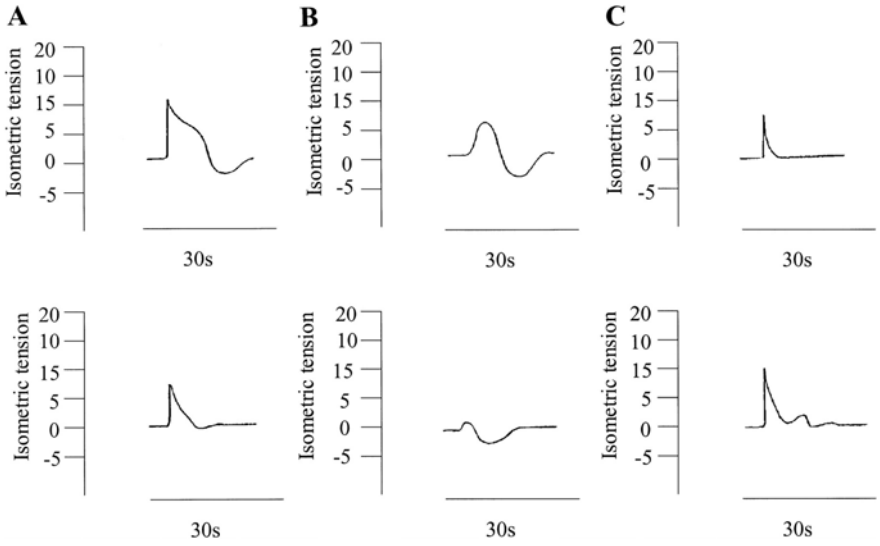

Fig. 4. Original tracings representing changes in isometric tension (mN) in bronchial preparations from normal and diabetic rats in response to field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli). The effect of atropine and capsaicin. Atropine (1 μM) was added directly to the organ bath, whereas capsaicin was applied as a sequential systemic pretreatment schedule to destroy the capsaicin-sensitive population of C fibers (see METHODS). *A*: untreated (control); *B*: after atropine; *C*: after capsaicin. *Top*: bronchial rings from normal animals. *Bottom*: rings from diabetic animals.

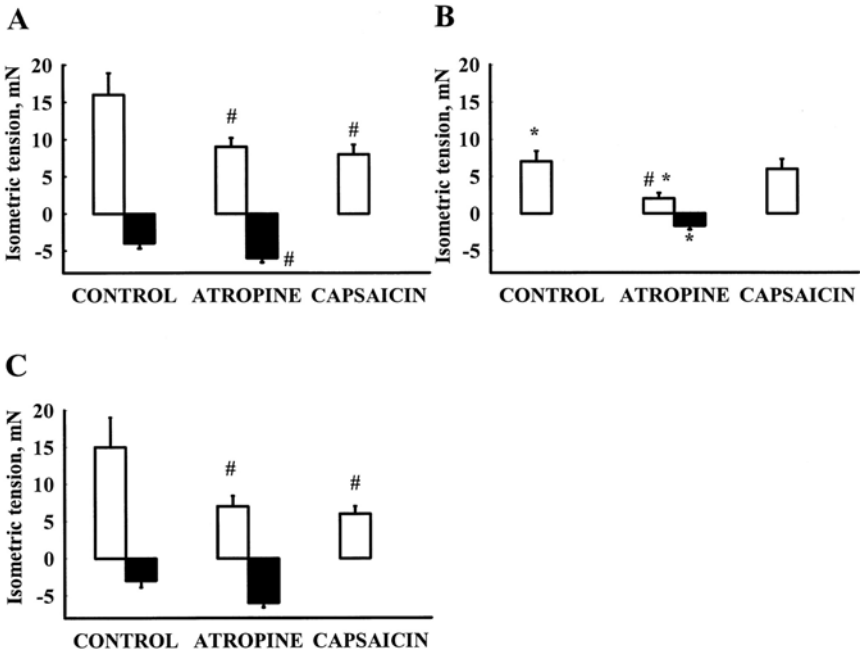

Fig. 5. Changes in isometric tension in bronchial preparations from normal (*A*), diabetic (*B*), and diabetic insulin-treated (*C*) rats in response to field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli). The effect of atropine and capsaicin. The data are means ± SD obtained with 6 preparations/group. # Significantly different from corresponding control at $P < 0.05$; * diabetic vs. normal at $P < 0.05$.

In rings from normal rats, both atropine (1 μM) and capsaicin desensitization significantly decreased contractions produced by FS (Fig. 5*A*). In addition, an augmented relaxation

response was seen after atropine, whereas pretreatment with capsaicin abolished the relaxation response to FS (Fig. 5*A*). In preparations from diabetic animals, capsaicin failed to significantly influence contractions by FS. The inhibitory effect of atropine on FS-induced contractions was striking. Atropine revealed a weak FS-induced relaxation response in preparations from diabetic animals (Fig. 5*B*). Preparations from the insulin-supplemented animals exhibited essentially similar responses to those seen in preparations from normal rats.

21.4.4.4 Sensory neuropeptide release.

FS-induced release of somatostatin, CGRP, and substance P was significantly attenuated in preparations from STZ-treated rats than in those from normal animals (Fig. 6). Insulin supplementation yielded complete restoration of FS-induced sensory neuropeptide release in STZ-treated rats. FS failed to elicit any significant neuropeptide release from preparations obtained from subgroups of rats that underwent pretreatment with capsaicin. Similarly, no neuropeptide release was seen with preparations from either main group preincubated with 1 μM TTX (Fig. 6).

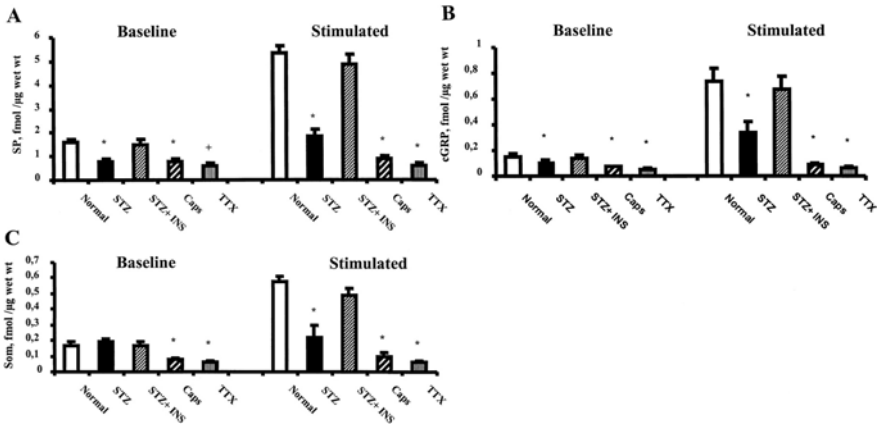

Fig. 6. Effect of field stimulation on sensory neuropeptide release from bronchial preparations from normal, STZ-, STZ + insulin (STZ+INS)-, capsaicin (Caps)-treated rats or from those exposed to 1 μM tetrodotoxin (TTX). The data are means ± SD obtained from 6 preparations/group. * Significantly different from corresponding "normal" values at $P < 0.05$. SP, substance P; CGRP, calcitonin gene-related peptide; Som, somatostatin.

21.4.4.5 Effect of sensory neuropeptides on FS-induced contractions.

Somatostatin and CGRP were without effect on isometric tension in mechanically precontracted rings in the absence of FS in preparations from either normal or diabetic animals. Substance P, however, produced a concentration-dependent increase in tension with maximum contraction of 12.3 ± 2.7 and 13.6 ± 3.4 mN with -log EC_{50} of 7.1 ± 0.2 and 7.0 ± 0.1 in preparations from normal and diabetic animals, respectively. Therefore, when the effect of substance P on FS-induced contractions was studied, the initial tension was reset each time to maintain a 12-mN resting tension before an FS challenge.
CGRP (up to 0.1 μM) and substance P (up to 1 μM) augmented the contractile response to FS in rings from both normal and diabetic rats. The potentiating effect of either neuropeptide on FS-induced increase in tension was significantly elevated in preparations from diabetic vs.

normal animals. Somatostatin decreased contractions by FS in both normal and diabetic preparations with a significantly attenuated inhibitory effect in bronchial rings from diabetic animals (Fig. 7).

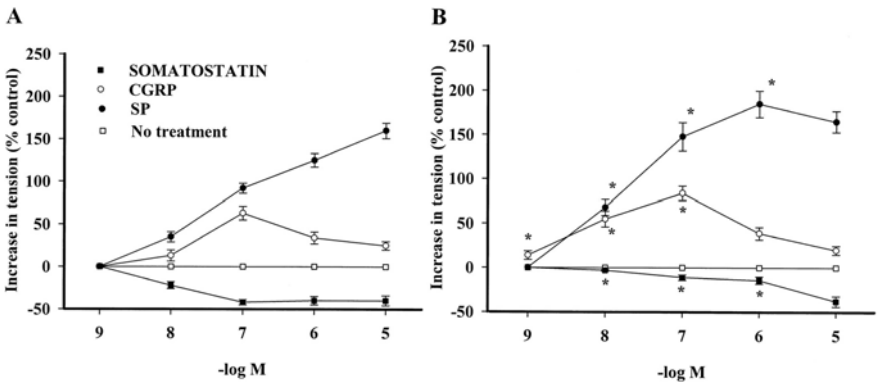

Fig. 7. Concentration-response effects of exogenous CGRP, SP, and somatostatin on contractile responses to electrical field stimulation (20 V, 0.1 ms, 20 Hz, 100 stimuli) in bronchial rings from normal (*A*) and diabetic (*B*) rats. The data are means ± SD obtained with 6 preparations/group. * Significant difference between diabetic (*B*) vs. corresponding normal (*A*) values at $P < 0.05$. The horizontal lines at 0% denote control (without treatment) responses to field stimulation in preparations from normal or diabetic animals. Positive values indicate an exaggerated contractile response (CGRP, SP), whereas negative values indicate alleviated contractions (somatostatin) after stimulation.

21.4.4.6 Antigen-induced trachea contraction.

In tracheal chains from nondiabetic ovalbumin-sensitized guinea pigs, cumulative increases in ovalbumin concentration in one-log unit steps produced concentration-dependent contractions with maximum values ~70% of those attained by 1 mM carbachol. The concentration-response curve for ovalbumin, however, was shifted to the right when the tracheal chains were prepared from diabetic animals (Fig. 8). The EC_{50} values for ovalbumin-induced contractions were 4×10^{-10} and 6×10^{-9} g/ml in chains from normal and diabetic animals, respectively. The maximum contractions by ovalbumin were also significantly decreased in preparations from diabetic animals (Fig. 8).

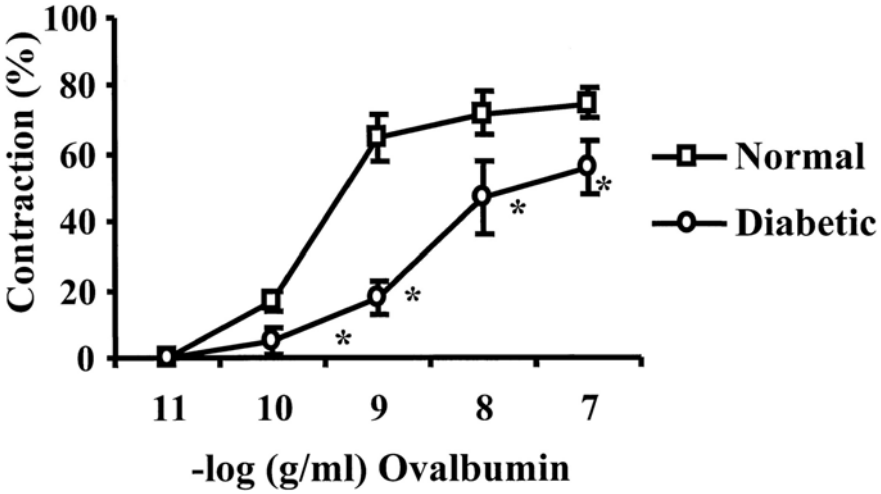

Fig. 8. Ovalbumin-evoked contractions of isolated tracheal chains prepared from ovalbumin-sensitized normal and diabetic guinea pigs. The contraction values are expressed as percentage of reference contraction induced by 1 mM carbachol. The data are means ± SD obtained with 6 preparations from 6 animals/group. * Diabetic vs. normal at $P < 0.05$

21.4.5 *Discussion*

These results show that STZ diabetes of 8-wk duration attenuates FS-induced bronchoconstriction/relaxation in vitro in rats and decreased bronchial contractions in response to antigen challenge in ovalbumin-sensitized guinea pigs. This attenuated bronchomotor response occurs in parallel with a significant decrease in the release of three sensory neuropeptides, such as that of somatostatin, substance P, and CGRP, in response to a highly standardized FS challenge in rats, which is the major original finding of the paper. The FS-induced bronchomotor response and the neuropeptide release were blocked by TTX, a fast Na^+ channel blocker; thus both can be considered to be of neural origin. These decreased responses were accompanied by a decline of femoral nerve conduction velocity in the STZ-treated animals. Because the nerve conduction velocity test is widely accepted as the "gold standard" of diabetic neuropathy (6, 15, 17), it is also confirmed that the diabetic animals suffered from sensory neuropathy 8 wk after STZ injection. Alternatively, because insulin supplementation restored both the deficient sensory neuropeptide release with reduced bronchomotor responses and femoral nerve conduction velocity abnormalities in STZ-treated animals, these alterations were considered to result from uncontrolled diabetes.
The STZ-treated rats exhibited characteristic features of type I diabetes in that they failed to gain weight, they suffered from hyperglycemia, and direct determination of fasting plasma insulin levels showed a substantial insulin deficiency. Moreover, the 8-wk diabetic state was associated with sensory neuropathy, which was reversible by insulin supplementation. Diabetic neuropathy is a demonstrable disorder, either clinically evident or subclinical, that occurs in the setting of diabetes mellitus without other cause of peripheral neuropathy (25). As an experimental approach, STZ-induced diabetes has been extensively used to study the pathogenesis and consequences of diabetic neuropathy (5). In this model, neuropathy, similar to that seen in the type I diabetes, typically involves detrimental changes in sensory, autonomic, and motor nerves (12, 27). As far as the mechanism of diabetic sensory neuropathy is concerned, a defective axonal transport of sensory neuropeptides in addition to

their decreased synthesis (24) is believed to be a critical initiating factor in degenerative distal neuropathies leading to severe microcirculatory changes (4, 9, 23). In addition, this possibly leads to a widespread deficiency in sensory effector function such as vasodilation, bronchomotility, or nonadrenergic, noncholinergic (NANC) contraction/relaxation (5, 15, 16, 17, 30, 33). In our main set of experiments, FS was used to study the effect of diabetes on nerve-mediated bronchoconstriction/relaxation. The results revealed that at least under our experimental conditions, the overall response to FS encompassed an initial atropine-sensitive contraction succeeded by a secondary longer-lasting contractile component resistant to atropine. These contractions were followed by relaxation, which also was atropine resistant. The latter two components, however, were blocked by prior systemic sequential capsaicin treatment, suggesting that they were of sensory neural origin. This treatment schedule has been shown to functionally deteriorate the majority of C fibers in rat (10). Experimental diabetes almost abolished the slow, atropine-insensitive contractile component and the relaxation component with less influence on the cholinergic response. Taking these results together with the decreased neuropeptide release from the bronchial preparations known to be densely innervated by CGRP and substance P-containing unmyelinated afferents that originate from the vagus nerve with cell bodies in the jugular, nodose, and dorsal root ganglia (28), we are not surprised that sensory neural dysfunction produced by diabetes was of significant influence on bronchomotor responses. Therefore, beyond the known impairment of cholinergic effector mechanisms in diabetes (3, 21), our present results strongly support the concept that a significant part of neural contractions of the bronchi is mediated by sensory neuropeptides, the release/effect of which is attenuated by diabetes. This is in accord with findings by Gamse and Jancso (11) and that by Gyorfi et al. (12), that neurogenic inflammation, another process underlain by the local effector function of sensory nerves, is attenuated by STZ-diabetes. However, when either substance P or CGRP was added to preparations from diabetic rats, its potentiating effects on FS-induced contractions were not impaired. Moreover, FS-induced contractions in the presence of either peptide were significantly elevated compared with those seen in muscle rings from healthy animals. This means that neither the effect of these peptides nor the contractile responsiveness of bronchial smooth muscle was impaired by diabetes. In addition, it is also confirmed that these neuropeptides play a significant regulatory role in neural contractions of the bronchi. However, the inhibitory effect of exogenous somatostatin on FS-induced contractions was diminished in preparations from diabetic vs. normal animals. Therefore, it is speculated that there was a sensitization to CGRP and substance P effects and a desensitization to the effect of somatostatin in rings from diabetic rats. Interestingly, the plasma level of somatostatin was found to be increased in diabetic animals, similar to that found previously in 4-wk STZ-diabetes in rats (20), which may explain the dissociated tissue sensitivity to the neuropeptides studied.

To the best of our knowledge, this work is the first to show that the attenuated bronchomotor response in insulin-deficient diabetes is related to a decrease in sensory neuropeptide release. Because NANC contractile agents such as substance P and tachykinins play an important role in neurogenic bronchoconstriction (18), this means that bronchi of diabetic animals are less prone to contract in response to neural and antigen challenges. Beyond providing some approach as to why bronchial hyperreactivity is attenuated in diabetes, the results also call attention to pharmacological exploitation of the sensory neuropeptide release/effect-bronchial smooth muscle contraction pathway to confer protection on patients at risk of bronchial hyperreactivity.

21.4.6 *Acknowledgements*

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21.4.7 *Footnotes*

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21.5 Impairment by lovastatin of neural relaxation of the rabbit sphincter of Oddi

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21.5.1 *Abstract*

We sought whether inhibition of cholesterol biosynthesis by lovastatin influenced the nitrergic relaxation response of the sphincter of Oddi. Rabbit sphincters of Oddi rings were tested for changes in isometric tension in response to field stimulation in the presence of 4 μM guanethidine and 1 μM atropine. Tissue samples were then analyzed for cAMP and cGMP content by radioimmunoassay for nitric oxide concentration by electron spin resonance and for vasoactive intestinal peptide and calcitonin gene-related peptide (CGRP) release by radioimmunoassay. Membrane $G_{s\alpha}$ protein was determined by Western blot analysis. Field stimulation relaxed the preparations with an increase in nitric oxide, cAMP and cGMP concentrations at increased calcitonin gene-related peptide and vasoactive intestinal polypeptide (VIP) release. Preparations from rabbits pre-treated with lovastatin (5 mg/kg/day intragastrically, over 5 days) contracted under the same conditions with an attenuated cGMP-increase at preserved increase in NO content and neuropeptide release. The relaxation was recaptured combining lovastatin with farnesol (1 mg/kg intravenously, twice a day for 5 days). The field stimulation-induced increase in cyclic nucleotides was also restored. Lovastatin decreased membrane $G_{s\alpha}$ protein content, which was re-normalized by farnesol. Farnesol treatment reinstates neurogenic relaxation of the sphincter of Oddi deteriorated by lovastatin possibly by normalizing G-protein coupling.

Author Keywords: Sphincter of Oddi; Nitrergic relaxation; Lovastatin; Farnesol

21.5.2 *Article Outline*

21.5.3 *Introduction*

Non-adrenergic, non-cholinergic (NANC) relaxation of the sphincter of Oddi is a prerequisite for normal delivery of bile into the duodenum (Pauletzki et al., 1993). This mechanism is essentially nitrergic in rabbits (Lonovics et al., 1994).

We have found that farnesol supplementation improves NANC relaxation of the rabbit sphincter of Oddi deteriorated by hypercholesterolemia (Szilvassy et al., 1998). This is in accordance with results by Roullet and Roullet that farnesyl analogues re-normalize vascular tone deteriorated by either hypercholesterolemia or inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a key enzyme in the mevalonate pathway independent of serum cholesterol levels. Therefore, the present work was to study if inhibition of HMG-CoA reductase by repeated administration of lovastatin decreased the NANC relaxation response in sphincters of Oddi preparations from otherwise healthy animals and as to whether this could be masked by farnesol supplementation.

A part of this work was presented in the annual meeting of the American Gastroenterological Association in San Diego, CA, 2000 (Sari et al., 2000).

21.5.4 *Methods*

21.5.4.1 Ethics

The experiments performed in the present work conform to European Community guiding principles for the care and use of laboratory animals. The experimental protocol applied has been approved by the local ethical committee of the Medical Universities of Debrecen, Szeged, and Pecs, Hungary.

21.5.4.2 Experimental groups

Sphincter of Oddi muscle rings were prepared from groups of adult male New-Zealand white rabbits (3000–3500 g) as follows: untreated animals ‘Group 1’, and from those treated with

lovastatin (5 mg/kg/day intragastrically, over 5 days; MEVACOR, MSD, Rahway, NY) 'Group2', or farnesol (1 mg/kg intravenously, twice a day for 5 days; Sigma, St. Louis, MO) 'Group 3', and from those given lovastatin+farnesol over 5 days 'Group 4'. Each group consisted of 23 preparations from 23 animals, six of which were used for measurement of isometric tension; five rings entered radioimmunoassay studies for determination of baseline cAMP and cGMP, eight rings were used for determination of tissue nitric oxide (NO) and four rings per group served for Western blot analysis of the membrane-associated G protein subunit $G_{s\alpha}$. Two additional groups (nine preparations in both) were instituted to test the effect of the solvent for farnesol and the placebo for lovastatin (four preparations for isometric tension measurements, three for cyclic nucleotide- and four for NO determinations per group). To reduce the number of experimental animals, field stimulation-induced changes in tissue cyclic nucleotide concentration were determined from the same preparations as those used for isometric tension measurements (see experimental protocol).

21.5.4.3 Isometric tension measurement

These have been described in detail elsewhere (Pauletzki et al., 1993). Biliary sphincter of Oddi muscle rings of approximately 6 mm length from adult male New Zealand white rabbits weighing from 3000 to 3500 g were prepared. The papilla Vateri was eliminated and the ampullary part of the muscle rings of approximately 3 mm length were mounted horizontally on two small L-shaped glass hooks of which one was connected to a force transducer (SG-O2, Experimetria, Budapest, Hungary) attached to a six channel polygraph (R61 6CH, Mikromed, Budapest, Hungary) for measurement and recording of isometric tension. The experiments were carried out in an organ bath (5 ml) containing Krebs bicarbonate buffer that was maintained at 37 °C and aerated continuously with carbogen. The initial tension was set at 10 milliNewton (mN) and the rings were allowed to equilibrate over 1 h. Atropine (1 μM) and guanethidine (4 μM) were continuously present (NANC solution). Changes in isometric tension in response to two consecutive trains of impulses of electrical field stimulation (40 stimuli 50 V, 0.1 ms and 20 Hz) were then studied.

21.5.4.4 Determination of cyclic nucleotide content in samples from isolated rabbit sphincter of Oddi

Tissue cGMP and cAMP contents were determined by means of radioimmunoassay as described (Szilvássy et al., 1994). Briefly, the muscle rings were snap frozen (to prevent cyclic nucleotides from breakdown by phosphodiesterases) in liquid nitrogen. The samples were then homogenized in 6% trichloroacetic acid. After thawing, the samples were processed at 4 °C. Sedimentation at 15,000×*g* for 10 min by means of a Janetzki K-24 centrifuge (Leipzig, Germany) was followed by extraction of supernatant with 5 ml water-saturated ether. The samples were evaporated under nitrogen, and assayed for cyclic nucleotide contents using Amersham radioimmunoassay kits (Les Ulis, France). The values were expressed as pmol/mg wet tissue weight.

Serum cholesterol level was determined as described (Szilvassy et al., 1995).

21.5.4.5 Membrane preparations and Western blot analysis

Sphincter of Oddi muscle rings were homogenized in ice-cold 50 mM Tris/HCl (pH 7.4 at 25 °C) containing 10 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 200 μg/ml bacitracin, 2 mM EDTA and 100 μM phenylmethylsulfonyl fluoride to prevent proteolysis. The

supernatant fraction resulting from centrifugation with 600×*g* for 10 min was re-centrifuged at 30,000×*g* for 15 min at 4 °C. The pellet was re-homogenized in fresh buffer and re-centrifuged. The final pellet was re-suspended in ice-cold assay buffer (50 mM Tris/HCl, 5 mM $MgCl_2$, pH 7.4 at 25 °C), and protein content was determined by Lowry's method using bovine serum albumin as a standard. Membrane prepararions were maintained at −80 °C for up to 2 weeks until utilized in assays. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blotting were performed using the procedure previously described by Miyamoto et al. (1992). In brief, membrane suspensions were dissolved in an equal volume of sample buffer containing 62.5 mM Tris/HCl (pH 6.8), 10% glycerol, 2% SDS, 5% mercaptoethanol and 0.0025% bromophenol blue and boiled for 5 min before application to the gel (7.5 μg protein per lane). After electrophoresis (40 mA for 100 min), the gels were soaked for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Proteins were transferred from the gel to pre-soaked nitrocellulose membranes at 180 mA over 90 min. The membranes were incubated for 2 h in 0.01 M Tris/HCl (pH 7.4)/0.9% NaCl containing 3% bovine serum albumin. Immunodetection was carried out by incubating the membrane with specific sheep antiserum recognizing $G_{s\alpha}$ diluted 1:2000 with the above buffer overnight at room temperature. The membranes were washed five times over 30 min. Membranes were then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-sheep IgG diluted 1:1000 with the above buffer. The antibody bound to nitrocellulose membrane was detected by the chromogenic substrate 4-chloro-1-naphtol. Immunoreactivity was also detected with the enhanced chemiluminescence Western Blot Detection System followed by exposure to Hyperfilm-enhanced chemiluminescence. Immunolabelled G proteins and the intensity of the specific bands were assayed by Soft Laser Scanning Densitometer (Biomed Instruments, USA)

21.5.4.6 Tissue NO determination by means of electron spin resonance

Nitric oxide content of freshly minced sphincter of Oddi tissue was measured using electron spin resonance spectroscopy after spin trapping with 55 mmol/l *N*-methyl-glucosamine-dithiocarbamate as described in details elsewhere. NO content was expressed as arbitrary units/mg tissue (Csont; Ferdinandy and Radak).

21.5.4.7 NANC neurotransmitter release studies

Calcitonin gene-related peptide (CGRP), concentrations were determined from 200 μl samples of organ fluid of the preparations by means of radioimmunoassay methods developed in our laboratories as described (Helyes and Nemeth). For radioimmunoassay determination of vasoactive intestinal polypeptide (VIP), we used commercial radioimmunoassay kits (Phoenix Pharmaceuticals, Belmont, CA, USA). Sampling was done prior to (resting values) and immediately after field stimulation (at maximum contraction/relaxation).

21.5.4.8 Sampling for cyclic nucleotide and NO determination

The muscle rings used for isometric tension measurements were used for radioimmunoassay studies as well, to determine field stimulation-induced changes in tissue cyclic nucleotide levels. Sampling was done so that the whole preparation exhibiting contraction/relaxation in response to field stimulation was placed in liquid nitrogen in 2 s subsequent to the maximum contractile response. For control to these series of rings served those which had not been subjected to field stimulation (resting values). The same sampling scheme was used for tissue NO measurement.

21.5.4.9 Drugs and chemicals

Beyond radioimmunoassay kits, all drugs and chemicals used in this study were purchased from Sigma except lovastatin and its placebo (MEVACOR, Merck-Sharp and Dohme, Hungaria Kft., Budapest, Hungary). Atropine and guanethidine were freshly dissolved in Krebs solution and added to the organ baths in 50 µl volume. Farnesol (3,7,11-trimethyl-2,2,10-dodecatrien-1-ol, mixed isomers) was diluted with 0.5 ml/kg body weight propylene glycol, therefore, propylene glycol was referred to as the solvent for farnesol.

21.5.4.10 Statistical analysis

The data representing changes in isometric tension and neuropeptide release expressed as means±standard deviation (S.D.) were evaluated by means of analysis of variance followed by a modified Student's *t*-test for multiple comparisons according to Bonferroni's method. Changes in tissue cyclic nucleotide and NO contents were evaluated by means of Student's *t*-test. Changes were considered statistically significant at *P* values smaller than 0.05.

21.5.5 *Results*

21.5.5.1 Isometric tension

The NANC relaxation response induced by field stimulation (20 Hz, 50 V, 0.1 ms, 40 stimuli) was converted to contraction in animals treated with lovastatin. Lovastatin–farnesol combination restored the normal NANC relaxation response (at the same field stimulation protocol). Farnesol was without effect on NANC relaxation by itself (Fig. 1). The NANC relaxation was not modified by the placebo for lovastatin or the solvent for farnesol.

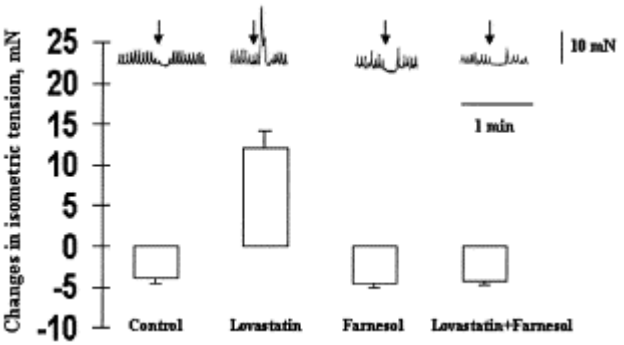

Fig. 1. Changes in isometric tension induced by electrical field stimulation (40 stimuli, 50 V, 0.1 ms and 20 Hz) in sphincter of Oddi preparations in vitro. The data are expressed as means±S.D. obtained with six preparations in each group. Positive values indicate contraction, negative values denote relaxation. The original tracings in the upper part of the figure represent characteristic responses to field stimulation in each particular group. The arrows show commencement of field stimulation.

21.5.5.2 Tissue NO content

In tissue samples of the sphincter Oddi, neither lovastatin nor farnesol or their combination influenced either baseline or post-stimulation intensity of specific spectra of NO-L-*N*-methyl-

glucosamine-dithiocarbamate complex assessed by electron spin resonance as compared to those from the untreated animals (Fig. 2A).

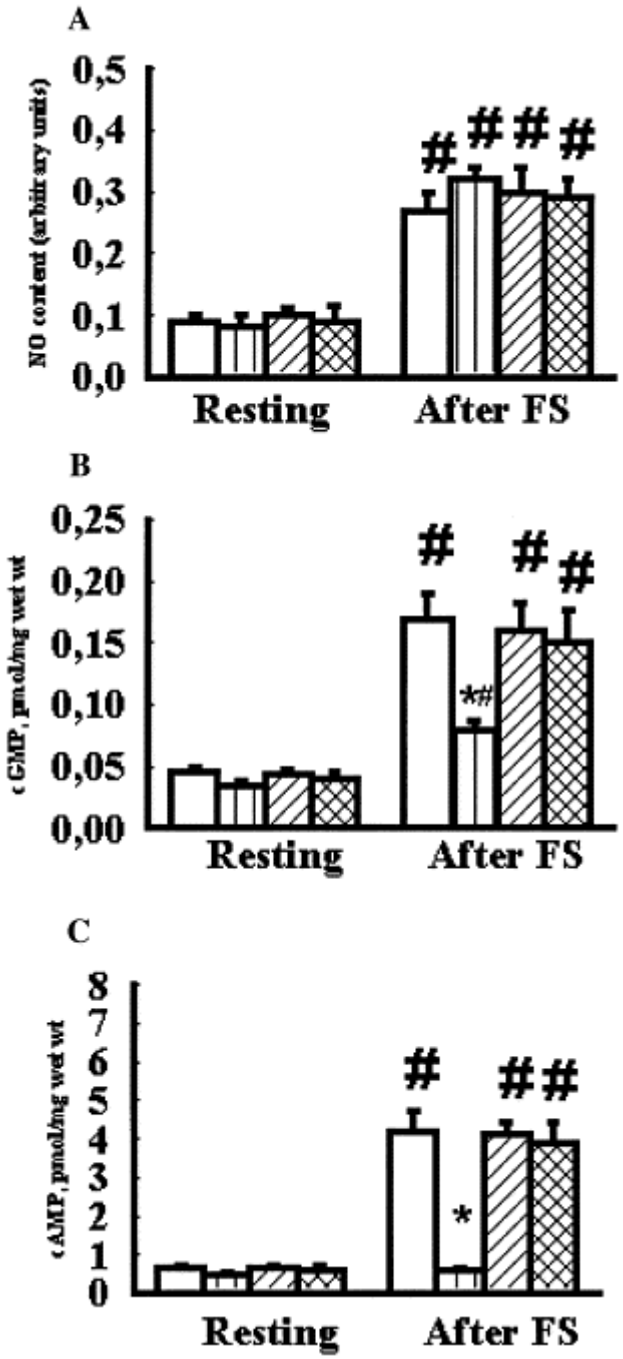

Fig. 2. Changes in tissue nitric oxide (NO, A) cGMP (B) and cAMP (C) content of sphincter of Oddi muscle rings in response to electrical field stimulation (0.1 ms, 50 V, 20 Hz, 40 stimuli). Open bars: rings from untreated animals; vertical line bars: lovastatin-treated animals; hatched bars: farnesol-treated; cross-hatched bars: lovastatin+farnesol-treated. Data are means±S.D. obtained with six rings from six animals. *: Lovastatin vs. untreated at $P<0.05$; #: stimulated vs. resting at $P<0.05$.

21.5.5.3 Changes in cyclic nucleotides

Field stimulation-induced NANC relaxation was accompanied by a significant increase in both cGMP and cAMP in preparations from the untreated animals. In muscle rings obtained

from the lovastatin-treated group, the increase in cGMP in response to field stimulation was much lower than that seen in sphincters from the untreated rabbits. Interestingly, field stimulation failed to increase cAMP in the lovastatin-treated group. Farnesol–lovastatin combination yielded complete restoration of the increase in both cyclic nucleotides in response to field stimulation (Fig. 2B and C). Farnesol, its solvent or the placebo for lovastatin was without effect.

21.5.5.4 NANC neurotransmitter release studies

Field stimulation induced a significant increase in VIP and CGRP concentration in organ fluid of the preparations as determined by means of radioimmunoassay. This was not modified by any of the treatments applied (Fig. 3A and B).

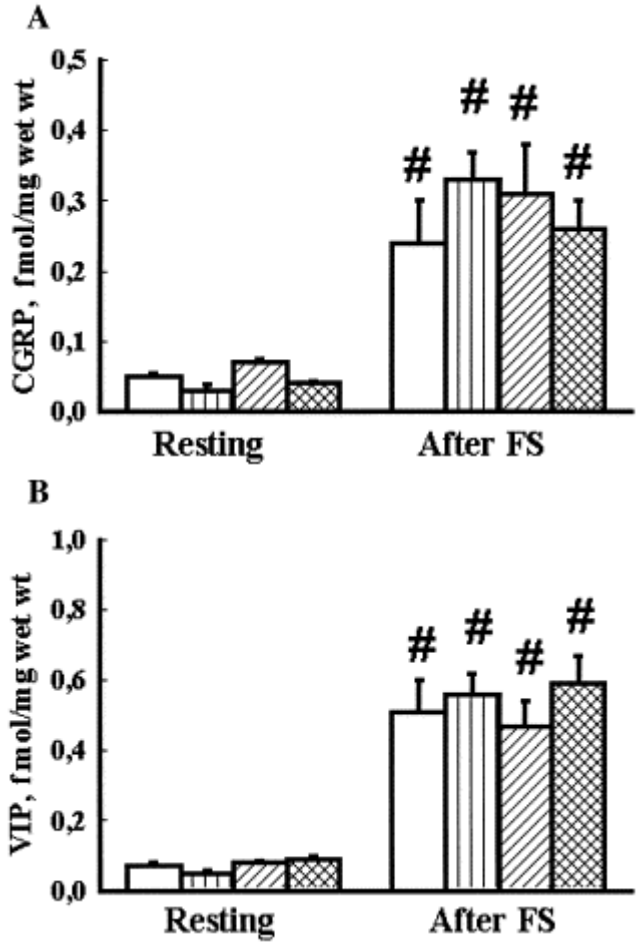

Fig. 3. Changes in calcitonin gene-related peptide (CGRP, A) and vasoactive intestinal polypeptide (VIP, B) release from isolated sphincter of Oddi muscle rings in response to electrical field stimulation (0.1 ms, 50 V, 20 Hz, 40 stimuli). Open bars: rings from untreated animals; vertical line bars: lovastatin-treated animals; hatched bars: farnesol-treated; cross-hatched bars: lovastatin+farnesol-treated. Data are means±S.D. obtained with six rings from six animals. *: Lovastatin vs. untreated at $P<0.05$; #: stimulated vs. resting at $P<0.05$.

21.5.5.5 Membrane composition of $G_{s\alpha}$ protein in the sphincter of Oddi

To estimate the effect of HMG-CoA inhibition on membrane particulation of G proteins in the sphincter of Oddi, quantification of $G_{s\alpha}$ protein, a representative of the membrane bound G protein complex, was done by immunodetection using specific $G_{s\alpha}$ antibody. The $G_{s\alpha}$ antiserum recognized a 57-kDa band, the density of which substantially decreased in membrane preparations from sphincter from animals treated with lovastatin (Fig. 4). Farnesol supplementation revealed re-normalization of $G_{s\alpha}$ density in muscle from the lovastatin-treated group. Farnesol was without effect (Fig. 4).

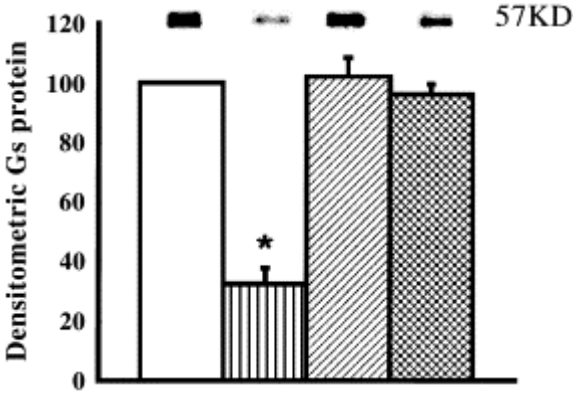

Fig. 4. Western blot analysis of $G_{s\alpha}$ subunit in rabbit sphincter of Oddi membranes. A total of 7.5 μg protein was loaded per lane. A representative gel obtained with preparations from untreated (open bars), lovastatin-treated (vertical line bars), farnesol-treated (hatched bars) and lovastatin+farnesol-treated (cross-hatched bars) animals is shown in the inset at the top of each corresponding bar. Densitometric results are expressed as a percent of density seen with preparations from untreated rabbits. Data are means±S.D. obtained with four preparations from four animals. *: Treated vs. untreated at $P<0.05$.

21.5.5.6 Serum cholesterol level

Serum total cholesterol level of 2.1±0.3 mmol/l decreased by 1.6±0.2 and 1.5±0.1 mmol/l ($P<0.01$ for both) by lovastatin and lovastatin+farnesol, respectively. Farnesol, the solvent for farnesol or the placebo for lovastatin were without effect.

21.5.6 *Discussion*

These results show that a 5-day treatment with lovastatin, a prototype of HMG-CoA inhibitors impairs neurogenic relaxation of the rabbit sphincter of Oddi, a mechanism shown to be nitrergic in nature (Lonovics et al., 1994). In fact, the relaxation response was converted to contraction in preparations from the lovastatin-treated animals similar to that seen after NO synthase inhibition (Lonovics et al., 1994). However, the present electron spin resonance studies reveal that lovastatin is without effect on NO synthesis since neither baseline nor post-stimulation tissue NO levels differed from those observed in muscle rings from untreated animals. Interestingly, the cGMP-increase in response to field stimulation was significantly lower in preparations from the lovastatin-treated animals than that in the untreated group, with loss of the stimulation-induced cAMP-increase after ‘lovastatin’. Treatment with farnesol attained a complete restoration of both neural relaxation and the cyclic nucleotide responses deteriorated by lovastatin.

It is widely accepted that NO, through cGMP synthesis, induces a sequence of protein phosphorylation that leads to smooth muscle relaxation (Moncada et al., 1991). The present results similar to that previously observed show that nitrergic relaxation of the rabbit sphincter of Oddi is accompanied by an increase in both cGMP and cAMP concentration (Szilvássy et al., 1997a). Theoretically, an increase in tissue cAMP concentration secondary to cGMP-increase might result from an inhibition of cAMP metabolism through inhibition of the enzyme type III (cGMP-inhibited cyclic nucleotide phosphodiesterase: PDE3) phosphodiesterase (Walter and Manganiello). However, the simultaneous increase in tissue cGMP and cAMP concentration in response to field stimulation can better be attributed to the co-release of NO and VIP (Grider and Jin, 1993) the latter of which is known to cause activation of specific membrane receptors coupled to a G protein complex for stimulation of adenylate cyclase and to increase cAMP (Altiere and Chakder). Notwithstanding, cAMP may increase in response to field stimulation due to adenylate cyclase stimulation by neurotransmitters released from intrinsic or sensory nerve terminals of the sphincter of Oddi other than VIP such as CGRP (Sand and Rasmussen). Moreover, in addition to its smooth muscle relaxing effect, NO has been proposed to stimulate the release of VIP (and perhaps other cAMP elevating agents) from enteric nerve terminals through presynaptic mechanisms (Allescher et al., 1996). VIP or CGRP, once released, facilitates further NO synthesis/release through cAMP-dependent pathways, thus, an interplay between NO and these NANC peptides underlie the increase in cGMP and cAMP in the sphincter of Oddi. Our present results are in good agreement with these observations, since the field stimulation-induced NANC relaxation was accompanied by a significant increase in NO, CGRP and VIP with an ensuing increase in both cAMP and cGMP.

The major original finding of the present work is that a 5-day treatment with the HMG-CoA reductase inhibitor lovastatin similar to dietary hypercholesterolemia (Szilvassy et al., 1996) abolished the NANC relaxation phenomenon at least under our experimental conditions. In the vasculature, functional defects have long been indentified in endothelial cells in hypercholesterolemia underlain by a deficiency in the release/some of the effects of NO both of which requiring the integrity of several G protein effector systems (Flavahan, 1992). To fulfil their biological function, G proteins must undergo a post-translation modification with farnesyl or geranylgeranyl moieties that enable them to associate with the membrane. The availability of these moieties, however, is reduced by both dietary hypercholesterolemia (Goldstein and Brown, 1990) and as a result of HMG-CoA reductase inhibition. We therefore considered possible that a 5-day treatment with the HMG-CoA reductase inhibitor lovastatin influenced nitrergic relaxation in the gastrointestinal tract in a similar way, resulting in a deficiency of both the release and effect of NO. The present results only partially support this assumption since the release of neither NO nor that of the two relaxant peptides was attenuated by lovastatin treatment. However, possibly due to its G protein dependence, no cAMP-increase was seen in sphincters from the lovastatin-treated group in response to field stimulation, whereas the G protein-independent cGMP formation was only partially impaired. The relative deficiency in cGMP formation after lovastatin may reflect the possible cAMP-dependent releasing effect of VIP and CGRP on NO, a mechanism confirmed in the vasculature (Sano et al., 2000). This latter mechanism, however, is not evident from our results, possibly due to the semi-quantitative nature of the electron spin resonance technique used for tissue NO determination, since approximately the same NO levels were seen with or without lovastatin. Furthermore, the smooth muscle relaxing effect of cGMP has been shown to involve a G protein-dependent activation of potassium channels that contributes to G protein-independent relaxation pathways (Ohno et al., 1993) (Fig. 5). Thus, the effect of NANC contractile mechanisms was unopposed during field stimulation in preparations from

the lovastatin-treated animals in part due to a deficiency in the interplay between cAMP and cGMP elevating agents supplemented with partial loss of cGMP effects. After combined treatment with lovastatin and farnesol, the normal NANC relaxation response was regained with an increase in tissue cGMP content in response to field stimulation.

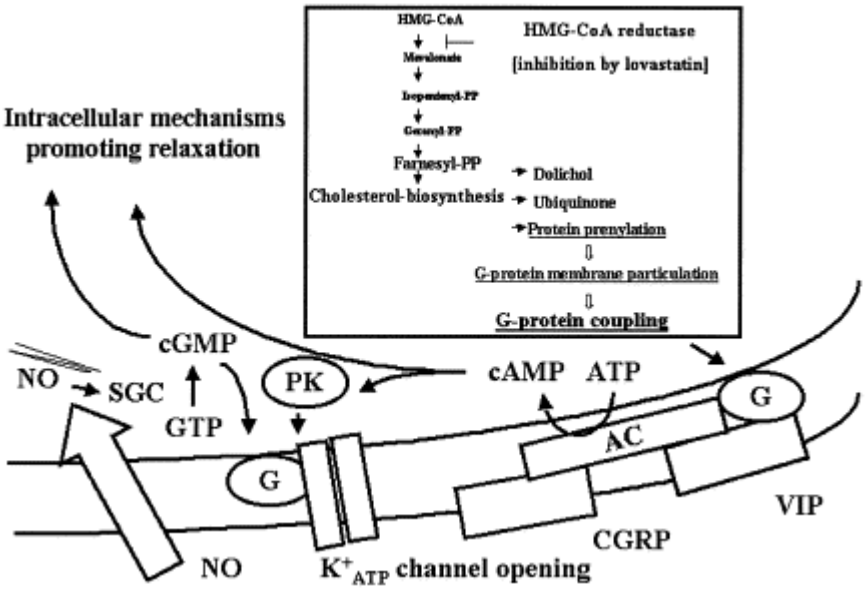

Fig. 5. Schematic diagram of the link between isoprenoid biosynthesis and mechanisms underlying NANC relaxation of the sphincter of Oddi. Inhibition of HMG-CoA by lovastatin decreases formation of non-cholesterol mevalonate products such geranyl-PP and farnesyl-PP important in protein prenylation, a process that plays a key role of membrane particulation of G proteins. Thus, inhibition by lovastatin of HMG-CoA reductase results in a deficiency in G protein-dependent signal transduction pathways such as cAMP formation and activation of potassium channels.

These results seem to support the assumption that a deficiency in the synthesis of non-cholesterol mevalonate products induced by either experimental hypercholesterolemia or pharmacological inhibition of HMG-CoA reductase, the key enzyme of cholesterol biosynthesis impairs NANC relaxation of the sphincter of Oddi. Farnesol supplementation, however, restored the relaxation response in both cases (Szilvassy et al., 1998).

Recent studies have suggested that non-cholesterol mevalonate products are implicated in the control of vascular tone and blood pressure (Roullet and Roullet). Since NANC relaxation is a pre-requisite for normal delivery of bile into the duodenum, a mechanism vulnerable to lovastatin, it is strongly suggested that non-sterol mevalonate-derived metabolites significantly contribute to the control of extrahepatic biliary tract motility as well (Szilvassy et al., 1998). This is supported by the fact that farnesol, the natural dephosphorylated form of farnesyl pyrophosphate that participates in protein farnesylation, recaptures the normal NANC relaxation function deteriorated by either hypercholesterolemia (Szilvassy et al., 1998) or HMG-CoA reductase inhibition. In certain clinical cases, however, a 1-month treatment with a low lovastatin dose (20 mg/kg in the evening), alleviated post-prandial right upper quadrant pain and improved the responsiveness to amylnitrite to enhance transpapillary bile flow as confirmed by results from hepatobiliary scintigraphy (Szilvássy et al., 1997b). The virtual contradiction may at least in part be explained by the difference in the degree of HMG-CoA reductase inhibition in different tissues in different species and that lovastatin may mask the

effect of hypercholesterolemia that deteriorates the relaxation function of the sphincter of Oddi by itself.

Beyond providing further evidence that non-sterol mevalonate products participate in widespread physiological regulatory mechanisms including sphincter of Oddi function, the results call attention to the non-lipid lowering effect of HMG-CoA reductase inhibitors which should be taken into account especially with long-term use of these drugs.

21.5.7 *Acknowledgements*

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21.6 Construction of a stable cell line uniformly expressing the rat TRPV1 receptor

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21.6.1 *Abstract*

We constructed and analyzed a new cell line called HT5-1, which stably expresses an enhanced green fluorescent protein-tagged version of the rat vanilloid receptor 1 (VR1/TRPV1). The fluorescent receptor allowed easy measurement of receptor expression and expression level-based purification of cells via fluorescence-activated cell sorting. The HT5-1 cell line was compared to cells transiently transfected with the fluorescent receptor, to cells expressing the native rat vanilloid receptor, and to isolated capsaicin-sensitive rat trigeminal sensory neurons. Fura-2 microfluorimetry measurements of the calcium influx upon capsaicin induction showed that, by contrast to transiently transfected cells, HT5-1 cells respond uniformly to the stimulation, due to the similar level of receptor expression in individual cells. HT5-1 cells showed similar behaviour to isolated trigeminal root ganglion neurons, including marked tachyphylaxis upon repeated capsaicin induction, and a lack of calcium ion release from intracellular storage sites.

Key Words: Capsaicin Receptor, TRPV1 Receptor Expression, Stable Cell Line, Green Fluorescent Protein, Flow Cytometry, Fluorescence-Activated Cell Sorting, TRG Neurons, Tachyphylaxis

21.6.2 *Introduction*

A systemic search for the postulated capsaicin receptor of polymodal nociceptive neurons [1, 2] led to the identification and cloning of a vanilloid

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gated cation channel which was also gated by noxious heat [3]. As a designation for this integrative nociceptive membrane protein [4], the acronym VR1 (vanilloid receptor 1) was used [3], but after the identification of several cation channels with similar structures, it was realized that there was a vanilloid subfamily of transient receptor potential (TRP) proteins, and VR1 was renamed TRPV1.

The identification of the TRPV1 gene meant it was possible to study the cloned receptor in transfected cells. The properties of the cloned TRPV1 receptor have been analyzed in markedly different expression systems ranging from cDNA injected into *Xenopus laevis* oocytes [3] and baculovirus infected Sf9 insect cells [5], to various transiently transfected mammalian cells including human embryonic kidney HEK293 [3], chinese hamster ovary CHO [6] and dorsal root ganglia derived F-11 cells [7]. TRPV1-expressing stable cell lines were mainly based on HEK293 cells [5, 8, 9], but cell lines derived from CHO [6, 10, 11] and C6 rat glioma cells [12] were also reported.

Differences in the expression systems led to observed discrepancies regarding the properties of the receptor protein and cellular responses involving the receptor, both between various transfected systems, and compared to isolated capsaicin-sensitive sensory neurons. For example, TRPV1 receptors expressed in HEK293 cells have a greater sensitivity to capsaicin and a lower sensitivity to resiniferatoxin than the native channels in DRG neurons [4, 13]. Furthermore, both the glycosylation state [14] and the subcellular location of the receptor [7,15] depend on the heterologous expression system. The elucidation of the complex responses exhibited by sensory neurons – like calcium influx-induced tachyphylaxis [16-19] – is hampered by TRPV1-mediated cellular responses being independent of extracellular calcium in some transfected cell lines [15], and some other systems not exhibiting tachyphylaxis at all [12]. A recent study directly comparing three different expression systems concluded that, for answering questions on cellular functions involving the TRPV1 receptor, stable cell lines that uniformly express it are preferable to transient transfections [12]. Unfortunately, only a limited variety of stable TRPV1-expressing cell lines have been reported. They are mainly based on HEK293 [5, 8, 9] or CHO cells [6, 10, 11], and some do not exhibit certain important characteristics of native TRPV1- expressing sensory neurons [12]. Therefore, other stable cell lines expressing the TRPV1 receptor and suitable to model capsaicin-sensitive neurons are needed. In this paper, we present our results from the construction and analysis of a new stable cell line called HT5-1, which is based on HT1080 human fibrosarcoma cells and expresses an enhanced green fluorescent protein-tagged (eGFP-tagged) version of the rat TRPV1 receptor. The fluorescent tag does not affect drug- induced receptor activation and can be used to measure the expression level in individual cells. Furthermore, the fluorescent tag allows the long-term maintenance of a uniform population of TRPV1-expressing cells via fluorescence-activated cell sorting (FACS), which can be used to isolate cells with a certain level of receptor expression. In the new cell line, the homogeneous receptor expression leads to uniform responses upon induction with capsaicin, in

contrast with the more varied reactions detectable in transiently transfected cells. HT5-1 cells exhibit similar properties to isolated TRG neurons including tachyphylaxis upon repeated induction and the lack of any response in a calcium-free extracellular medium.

21.6.3 *Materials and methods*

21.6.3.1 Construction of the TRPV1 expression vectors

Total RNA was isolated from the trigeminal ganglia of newborn rats with the High Pure RNA isolation kit (Roche Inc.). The RNA was reverse transcribed and amplified in one step with the Titan One Tube RT-PCR system (Roche Inc.) using primers which bind to positions 58-78 and 2626-2647 on the rat TRPV1 mRNA (Genbank #AF029310). The parameters of the PCR reaction were: 30 minutes reverse transcription at 50ºC, followed by 15 cycles of 10 seconds denaturation at 94ºC, then 30 seconds annealing at 56ºC and 2 minutes extension at 68ºC, followed by 20 cycles as the extension time was increased to 3 minutes. The reaction was completed with a 7-minute final extension at 68ºC. The RT- PCR reaction gave a single 2.6-kb sized band on agarose gel electrophoresis.
The amplified cDNA was isolated from the gel, treated with Klenow polymerase and cloned into the SmaI site of pGEM-3zf plasmid (Promega Inc.). Despite the presence of the proofreading Tgo polymerase in the PCR reaction, sequencing showed that all the clones had mutations compared to the published rat capsaicin receptor sequence (Genbank #AF029310). We selected the clone in which the mutations did not affect the amino acid sequence of the protein (the mutations in this clone were A to G transitions at positions 86, 2597 and 2614). The TRPV1 cDNA was moved from this clone into mammalian expression vector pEGFP-N1 (Invitrogen Inc.) between the EcoRI and SalI cloning sites. Two different expression vectors were constructed. The vector called pTRPV1 expresses the native receptor cDNA, because the eGFP coding region between the BssHI and NotI sites was removed from the original vector. In the second vector, called pTRPV1eGFP, the C terminal of the receptor is fused to the enhanced green fluorescent protein through the sequence 5' GGGGCCCGGGATCCACC GGTCGCCACC 3', which encodes the ten-amino acid linker peptide GARDPPVAT.

21.6.3.2 Expression of the rat TRPV1 receptor in transfected human cells

Human fibrosarcoma cell line HT1080 [20] was used to express the rat TRPV1 receptor. The cells were grown in DMEM medium containing 10% fetal calf serum and supplemented with non-essential amino acids and antibiotics. 5 μg of the vectors were transfected into $0.5x10^6$ cells on 35-mm dishes using the Fugene 6 transfection reagent from Roche Inc. according to the protocol supplied by the manufacturer. The cells were analyzed 40 hours after transfection. Stable cell lines were created by plating two days after the transfection of 10^6 transfected cells ($5x10^4$ cells/10-cm dish) in a medium containing 500 μg/ml of the G418 drug. 14 days later, single colonies were

picked, expanded and checked for the presence of the functional receptor via fura-2 microfluorimetry. The presence of the TRPV1 mRNA in the transfected cells was also checked by RT-PCR. Similar conditions were used as before, but a 519-bp part of the target was amplified using forward and reverse primers which bind to positions 58-78 and 557-577, respectively.

21.6.3.3 Western blotting

TRPV1 protein expression in the transfected cells was analyzed via Western blotting according to standard procedures. Two days after transfection, 10^6 cells were collected and lysed in 200 □l of SDS sample buffer. 25 □l of the samples were electrophorised on a vertical 6% polyacrylamide gel made without SDS, in electrophoresis buffer containing 0.1% SDS. After electrophoresis, the gel was electroblotted overnight to a nitrocellulose membrane (Bio-Rad Laboratories Inc.). A goat polyclonal antibody raised against the amino terminus of the rat vanilloid receptor (sc-8670) was used as the primary antibody at 1:1000 dilution. The secondary antibody was a 1:1000 diluted alkaline phosphatase-linked donkey anti-goat antibody (sc-2037). Both antibodies and the compatible Cruz Marker protein molecular weight ladder were obtained from Santa Cruz Biotechnology Inc. The alkaline phosphatase was visualized directly on the nitrocellulose membrane via the Western Blue colorimetric assay from Promega Inc.

21.6.3.4 Flow cytometric analysis of TRPV1eGFP fusion protein expression

TRPV1eGFP-expressing cells were trypsinized, washed once with PBS and suspended in PBS at 10^6 cells/ml. Green fluorescence was measured using the FL1 detector on a BD FACSCalibur flow cytometer (BD Biosciences Inc.). The mean fluorescence of untransfected cells was around 40 units. Cell separation experiments were performed on a BD FACSVantage SE instrument (BD Biosciences Inc.) as recommended by the manufacturer [21]. Cells with green fluorescence higher than 100 units were isolated. The sorted cells were grown in the presence of 100 units/ml penicillin, 100 μg/ml streptomycin and 500 μg/ml G418 for a week, to prevent infection of the cell culture.

21.6.3.5 Radioactive calcium-45 uptake experiments

The experiments were essentially performed as described previously [22]. Briefly, ~2500 HT5-1 cells/well were plated in 15 μl cell culture medium onto Microwell Minitrays from Sigma Inc. On the following day, the cells were washed 5 times with HEPES (10 mM, pH 7.4) buffered magnesium and calcium-free Hank's balanced salt solution supplemented with 2 mM calcium chloride. Then the cells were incubated in 10 μl of the same buffer containing the desired amount of drug and 200 μCi/ml calcium-45 isotope (1.3 Ci/mmole, Ammersham) for 10 min at 37ºC. After incubation, the cells were washed 5 times with assay buffer (5 mM KCl, 2 mM $MgCl_2$, 12 mM glucose, 10 mM HEPES (pH 7.4), 137 mM sucrose, 5.8 mM NaCl, and 0.75 mM $CaCl_2$), the residual buffer was evaporated, the retained isotope was collected in 10 μl 0.1%

SDS and the radioactivity was measured in 2 ml scintillation liquid in a Packard Tri-Carb 2800 TR scintillation counter.

21.6.3.6 Preparation of TRG neurons

Primary cultures of trigeminal neurons were made from the trigeminal ganglia of 1- to 7-day old Wistar rat pups with collagenase and deoxyribonuclease I treatment as described previously [23]. Neurons were grown on poly-D-lysine- coated glass cover slips in cell culture medium composed of 85 ml DMEM, 5 ml horse serum, 5 ml fetal bovine albumin, 5 ml newborn calf serum, and the appropriate amount of antibiotics. Nerve growth factor (5 ng/ml) was added every second day.

21.6.3.7 Fura-2 microfluorimetry experiments

Microfluorimetry experiments on TRG neurons and transfected cells were performed as reported [22]. The cells were grown on glass cover slips, incubated for 30 minutes in incubation buffer (122 mM NaCl, 3.3 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 10 mM glucose, 25 mM HEPES, pH 7.4) containing 1 μM fura-2 AM (Molecular Probes Inc.). Dye-loaded cells were examined at room temperature in ECS buffer (160 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.3). In some experiments, the cells were kept in calcium-free ECS buffer containing 3 mM EGTA instead of calcium chloride. The analysis was performed on an Olympus BX50WI upright microscope alternately at 340 and 380 nm light generated by a monochromator (Polychrome II., Till Photonics Inc.), and the fluorescence emitted at 510 nm from individual cells was detected with a digital camera (CCD, SensiCam PCO). The data was recorded and the 340/380 nm fluorescence ratio was analyzed using Axon Imaging Workbench 2.1 software (Axon Instruments Inc.) on a PC.

21.6.3.8 Chemicals

The cell culture medium, fetal bovine serum and other cell culture supplements were Gibco products obtained from Invitrogen, Inc. Unless stated otherwise, the chemicals used in the experiments were obtained from Sigma-Aldrich Inc. TRPV1 antagonist SC-0030, also denoted JYL1421 ([N-(4-tertbutylbenzyl)-N'-[3-fluoro-4-(methylsulfonylamino)benzyl]-thiourea) [24], was provided by Professor Uhtaek Oh.

21.6.4 *Results and discussion*

21.6.4.1 Cloning and transient expression of the rat TRPV1 receptor

The TRPV1 receptor cDNA was RT-PCR amplified using total RNA prepared from the trigeminal ganglia of 1- to 7-day old rat pups, as described above. Two vectors were constructed, one expressing a C-terminal eGFP-tagged version of the receptor, called pTRPV1eGFP (Fig. 1A), and the other expressing the native receptor, called pTRPV1.

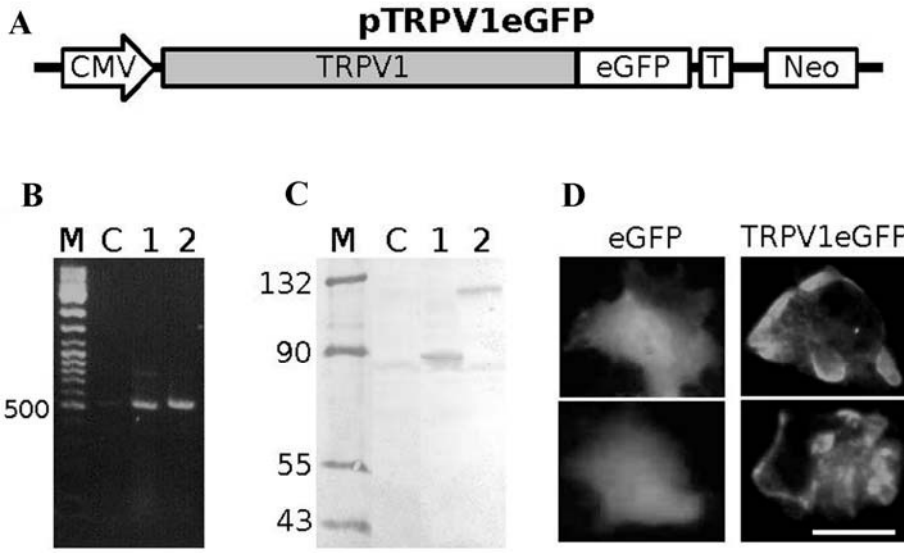

Fig. 1. The expression of the eGFP-tagged rat TRPV1 receptor in human HT1080 cells.
A - A schematic representation of the pTRPV1eGFP expression vector. CMV: cytomegalovirus promoter, TRPV1: capsaicin receptor, eGFP: enhanced green fluorescent protein, T: SV40 transcription terminator, Neo: neomycin resistance gene.
B - Detection of the expressed TRPV1 mRNA by RT-PCR in transiently transfected cells. M: molecular weight ladder, C: untransfected control, 1: transfected with pTRPV1 vector, 2: transfected with pTRPV1eGFP vector. The 500 bp band of the ladder is indicated. The expected size of the amplified DNA is 519 bp. C - Detection of the expressed TRPV1 protein by western blotting in transiently transfected cells. M: molecular weight marker, C: untransfected control, 1: transfected with pTRPV1 vector,
2: transfected with pTRPV1eGFP vector. The molecular weight of the marker bands are indicated in kDa. The expected size of the TRPV1 protein in sample 1 is 95 kDa, while the expected size of the TRPV1eGFP fusion protein in sample 2 is 122 kDa.
D - Detection of the expressed TRPV1eGFP protein by fluorescent microscopy. The left
column shows two photos of cells expressing the eGFP protein. The right shows two photos of cells expressing the TRPV1eGFP fusion protein, where the fluorescence is uneven and mainly localized to membrane structures of the cell. The white line is the 25- □m scale bar.

The human fibrosarcoma-derived cell line HT1080 [20] was selected to express the cloned receptor. HT1080 cells are resilient, have a flattened shape, tend to form monolayers, and attach strongly to untreated glass surfaces, and therefore are convenient to work with. They also have a stable karyotype with a chromosome number of 46±2, which is advantageous in maintaining long-term transgene expression. HT1080 cells were transiently transfected with the vectors, and TRPV1 mRNA expression was detected via RT-PCR (Fig. 1B). The expected 519-bp long PCR band appears in both the pTRPV1 and the pTRPV1eGFP transfected samples, indicating the presence of TRPV1 mRNA.

The expression of the TRPV1 protein was analyzed by Western blotting. As can be seen in Fig. 1C, in the pTRPV1 transfected sample, the immunoreactive band appears at ~90 kDa, which is somewhat lower than the expected 95 kDa. This is probably due to slight variations in the electrophoretic conditions. In the sample from the pTRPV1eGFP-transfected cells, a band appears at the expected 122 kDa range indicating the presence of the receptor-eGFP fusion protein. Expression of the TRPV1eGFP fusion protein could also be detected in living cells using fluorescent microscopy (Fig. 1D). In control cells transfected with the pEGFP-N1 plasmid, the eGFP protein is evenly distributed in the cytoplasm. By contrast, the TRPV1eGFP fusion protein is mainly located in the membrane structures of the cell, thus showing uneven distribution. The fluorescent tag on the fusion protein made it possible to monitor and quantify receptor expression via flow cytometry in later experiments.

21.6.4.2 Construction of stable cell lines expressing the rat TRPV1 receptor

Stable cell lines expressing the TRPV1 and the TRPV1eGFP proteins were obtained by G418 drug selection, after transfections with the appropriate vectors. The presence of a functioning vanilloid receptor was confirmed in selected clones via a fura-2 based microfluorimetry assay measuring capsaicin-induced calcium ion influx in individual cells. We examined several clones, and selected two for further experiments: HT4-13, expressing the native TRPV1 receptor, and HT5-1, expressing the TRPV1eGFP fusion protein. It seems that the eGFP tag at the C-terminal end of the receptor does not significantly alter the pharmacological properties of the TRPV1 receptor [15]. To confirm these results, we compared the responses of TRPV1 and TRPVeGFP receptors to treatment with the agonists capsaicin and resiniferatoxin, and the antagonists 5'- iodoresiniferatoxin, SC-0030 and ruthenium red. Drug-induced calcium influx was measured using the radioactive calcium-45 ion uptake assay. The measured activities relative to the 100 nM capsaicin-induced responses (100%) in each cell line are presented in Fig. 2. Both the 100 nM capsaicin and the 10 nM resiniferatoxin treatments induced a similar level of calcium ion uptake in both cell lines, though the resiniferatoxin treatment was ~25% less effective. When cells were preincubated for 15 minutes with 10 μM of the antagonists 5'- iodoresiniferatoxin, SC-0300 or ruthenium red, the 100 nM capsaicin treatment failed to induce calcium ion uptake above the level of the untreated control in the two cell lines. There was no significant difference between the responses obtained with HT4-13 cells expressing the native TRPV1 receptor and HT5-1 cells expressing the TRPV1eGFP fusion protein with any of the tested drugs. Data obtained previously with HT5-1 cells in dose-response experiments showed that the EC_{50} values are 36 nM for capsaicin, 1.5 nM for resiniferatoxin, and 1.8 μM for N-oleoyldopamine [22]. These results indicate that the TRPV1eGFP fusion protein can be used to model the behaviour of the native vanilloid receptor and has the advantage that receptor expression can be monitored by fluorescent methods.

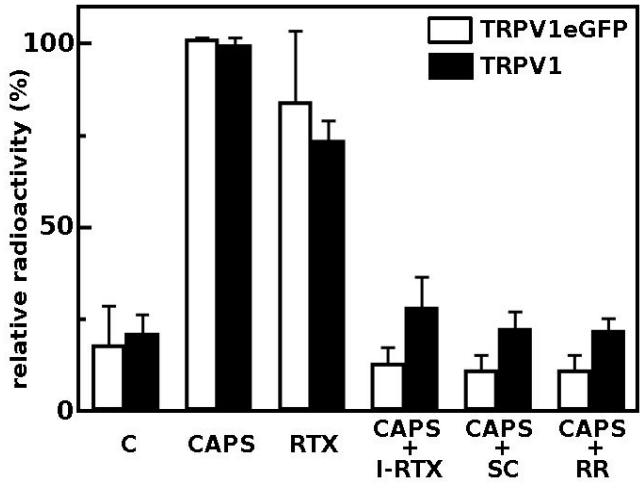

Fig. 2. Comparison of calcium influx in the TRPV1eGFP-expressing cell line HT5-1 (white bars) and the TRPV1-expressing cell line HT4-13 (black bars), as measured using the radioactive calcium-45 uptake method. 100 nM capsaicin (CAPS) and 10 nM resiniferatoxin (RTX) were used as agonists and 10 μM 5'-iodoresiniferatoxin (I-RTX), drug SC0030 (SC) and ruthenium red (RR) were used as antagonists against 100 nM capsaicin. C are the untreated control cells. The measured CPM values were normalized to the intensities obtained with 100 nM capsaicin (100%) in both cell lines and the relative intensities are presented. Experiments were repeated three times and the error bars represent the standard error of mean.

21.6.4.3 FACS purification of the HT5-1 cell line

As common with stably transfected cell lines, the ratio of cells expressing the transgene decreased during passaging in both the HT4-13 and the HT5-1 cell lines, even when continuous G418 drug selection was applied. This problem could be mitigated by the presence of the fluorescent tag on the receptor in the HT5-1 cell line, which made it possible to separate receptor-expressing cells by fluorescence-activated cell sorting. The flow cytometer was set up so that untransfected cells had a mean fluorescence of around 40 units, while the mean fluorescence of TRPV1eGFP-expressing cells was around 500 units as measured with an FL1 detector. During the purification of cell line HT5-1, cells showing fluorescence higher than 100 units were isolated. In Fig. 3 a flow cytometry histogram showing receptor expression in the cell population before and after sorting is presented. The ratio of green fluorescent cells over the 100 unit threshold falling in the M1 region could be increased from 19% to 88% via FACS purification. This data indicates that in the HT5-1 cell line, the expression of the TRPv1eGFP receptor can be measured in individual cells, and a population of cells with a certain level of receptor expression can be isolated. These properties facilitate the long-term maintenance of HT5-1 cells uniformly expressing the receptor, which makes quantitative analysis of receptor functions more straightforward, as demonstrated in the next experiments.

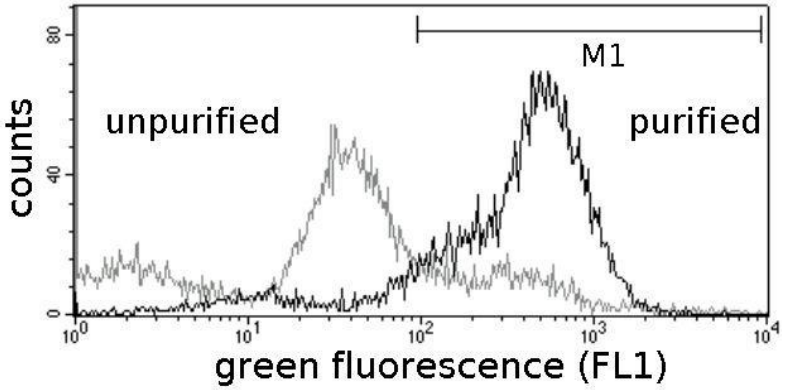

Fig. 3. Fluorescence-activated cell sorting was used to purify HT5-1 cells expressing the TRPV1eGFP protein. A flow cytometry histogram of the cell populations before and after sorting is presented. Gates were set up to isolate cells with a fluorescence intensity above 100 units: that value was chosen to indicate receptor expression. The marked region (M1) contains only 19% of the cells in the unpurified sample, but 88% of the cells in the purified population, representing an over four-fold increase in the ratio of receptor expressing cells in the population after sorting.

21.6.4.4 Comparison of transiently transfected cells and the HT5-1 stable cell line

Transiently transfected cells express the TRPV1 receptor at various levels; this phenomenon was proposed to be the reason for the observed discrepancies in individual cellular responses within the population upon receptor stimulation [12]. Using flow cytometry analysis, we compared the level of TRPV1eGFP expression in stable cell line HT5-1 and in HT1080 cells transiently transfected with the pTRPV1eGFP vector. 35% of the transiently transfected cells and 85% of the HT5-1 cells expressed the receptor. Histograms showing only the distribution of positive cells with fluorescence >80 units are presented in the upper part of Fig. 4. It can be seen that 75% of the transiently transfected cells express a moderate amount of the receptor (region M1) while 25% show a significantly higher expression level (region M2). The histogram also illustrates that the transiently transfected cell population exhibits a broad spectrum of expression levels which varies by more than an order of magnitude between individual cells. By contrast, the HT5-1 cell line displays a more uniform, moderate level of expression, with 99% of the positive cells falling in region M1, and no over-expressed population (M2) (Fig. 4B). Simultaneous fura-2 microfluorimetry analyses of 10 responsive cells showed that transiently transfected cells display a heterogeneous response upon 100 nM capsaicin induction, differing in both the amplitude and the duration of the intracellular calcium influx between individual cells (Fig. 4C). The difference between the lowest and highest amplitudes of the responses was 0.7 units, while the duration of the responses ranged from 50 seconds to more than 150 seconds. In two cells, the intracellular calcium ion level did not return to its prestimulation level during the observed time period. By contrast, in cell line

HT5-1, where individual cells express the receptor at the same level, more homogeneous responses were observed. On analysis of the simultaneously recorded responses of 11 cells, less than 0.2 units of difference was found between the amplitudes, and the duration of the responses fell between 75 and 100 seconds.

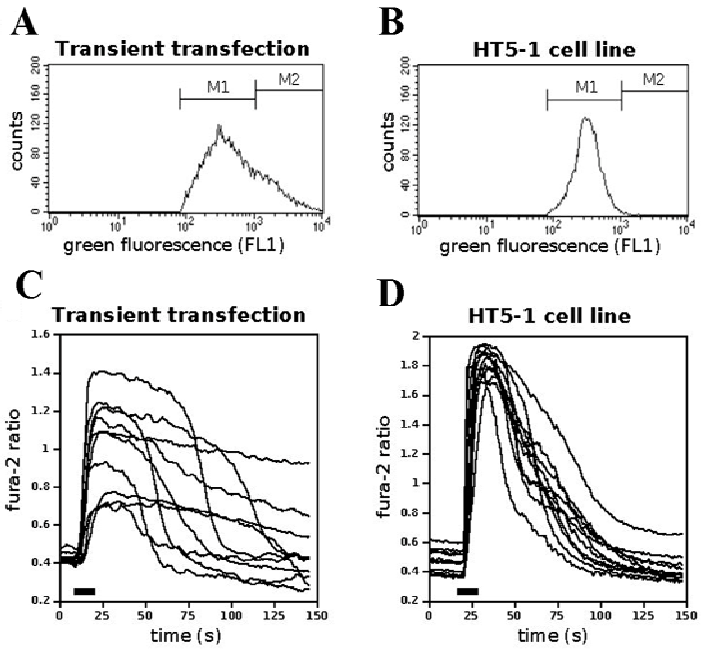

Fig. 4. A comparison of transiently transfected cells and stable HT5-1 cells, both expressing the TRPV1eGFP protein. The upper part of the figure shows the analysis of receptor expression by flow cytometry for transiently transfected (A) and stable HT5-1 cells (B). Only cells showing a fluorescence of over 80 units are included in the histograms. In the transient transfection, 75% of the cells fall in the region marked M1 and 25% in M2, while 99% of the HT5-1 cells fall in the M1 region, indicating a more uniform expression. In the lower part of the figure, the response of the same population of cells to capsaicin treatment is presented. Individual fura-2 signals indicating the free intracellular calcium ion levels of 10 cells from a single transient transfection (C) and 11 cells of the HT5-1 cell line (D) are shown. The black bar indicates the point at which the cells were exposed to 330 nM capsaicin for 10 seconds. The HT5-1 cells present a uniform response, while in the transiently transfected cells, both the intensity and the duration of the responses differ greatly.

21.6.4.5 Similar behaviour displayed by TRG neurons and HT5-1 cells

We compared HT5-1 cells to native trigeminal root ganglion neurons that had been freshly isolated from 1- to 7-day old rats. The results of fura-2 microfluorimetry experiments, averaging responses from several cells, are

resiniferatoxin induction, showing a lasting elevation in the level of intracellular calcium ions (panels A and B).When stimulated with 1 μM capsaicin plus 3 mM EGTA solution for 60 seconds in a calcium-free extracellular buffer containing 3 mM EGTA, neither TRG neurons nor HT5-1 cells showed any response (panels C and D). The same cells responded with a marked increase in the intracellular calcium ion level when a 10-second treatment of 1 μM capsaicin plus 1 mM calcium chloride was applied later. Longer capsaicin treatment (up to 3 minutes) was also ineffective with HT5-1 cells in a calcium-free extracellular medium (data not shown). Upon repeated inductions with 330 nM capsaicin, both the TRG neurons and HT5-1 cells showed marked tachyphylaxis, resulting in decreased responses upon the second and third stimulations. The second peak was 60% and the third peak was 38% of the amplitude of the first for the TRG neurons (Fig 5E.). HT5-1 cells exhibited a similar degree of tachyphylaxis, showing a 56% sized second and a 41% sized third peak (Fig. 5F). Tachyphylaxis was also measured via the radioactive calcium-45 uptake method for HT5-1 cells using 10-minute long treatments with 100 nM capsaicin, resulting in a 31% sized second and a 18% sized third response peak (data not shown). This data indicates that the HT5-1 cell line can serve as a useful model for capsaicin-sensitive sensory neurons, even when complex cellular responses like tachyphylaxis are involved.

In this paper, we reported on the construction and analysis of a new stable cell line called HT5-1, which expresses an eGFP-tagged version of the rat TRPV1 receptor and has several advantages over some of the previously described TRPV1 expression systems. The fluorescent version of the TRPV1 receptor behaves similarly to the native receptor when induced by capsaicin and resiniferatoxin, or blocked by the antagonists iodoresiniferatoxin, ruthenium red and SC0030 (JYL1421) [24]. The EC_{50} values of 36 nM for capsaicin and 1.5 nM for resiniferatoxin fall in the range of previously published figures obtained with different TRPV1-transfected cell systems, ranging from 34 nM [25] to 711 nM [3] for capsaicin and from 0.1 nM [15] to 39 nM [3] for resiniferatoxin. These results indicate that the C-terminal eGFP tag does not significantly alter the pharmacological properties of the receptor; this concurs well with previous observations [15, 26]. The length of the C-terminal end of the TRPV1 receptor influences thermal sensitivity, as progressive shortening of the tail decreases the temperature threshold for channel activation [27]. Further analysis is required to determine whether the heat sensitivity of the receptor is altered in HT5-1 cells due to the increased length of the C-terminal end of the TRPV1eGFP fusion protein.

Studying TRPV1 receptor function in transiently transfected cells has the disadvantage that the system varies greatly between transfections, and even in a single transfection, individual cells with different levels of receptor expression are present. This leads to heterogeneous responses in the cell population, making the interpretation of the obtained data more difficult [12]. The HT5-1 cell line

has the advantage that receptor expression can be monitored, and a homogeneous population of cells with approximately the same level of expression can be isolated using fluorescence-activated cell sorting. Compared to transiently transfected cells which over-express the receptor, purified HT5-1 cells with moderate receptor expression exhibit a much more uniform response.

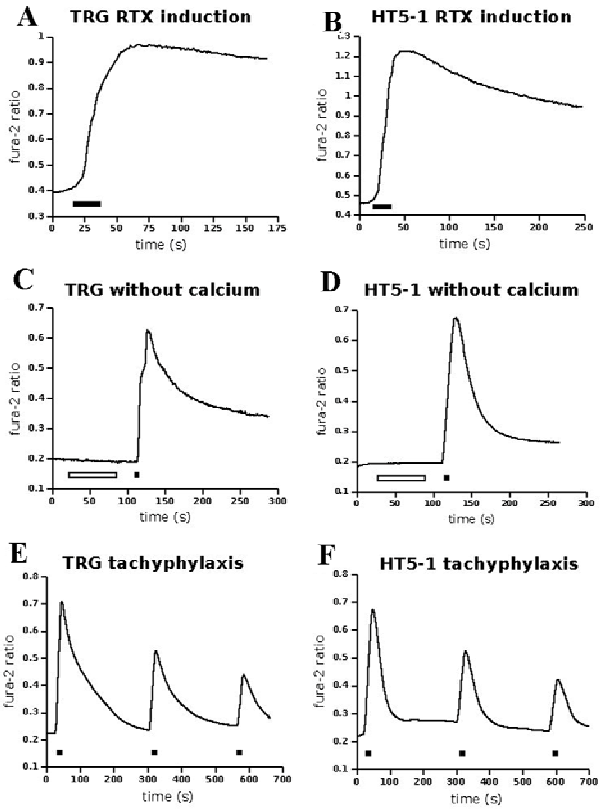

Fig. 5. A comparision of rat capsaicin-sensitive TRG neurons and HT5-1 cells, performed via microfluorimetry. Averaged fura-2 signals indicating the intracellular calcium ion levels of 2-16 cells upon different inductions are presented. Both TRG neurons (n=12) (A) and HT5-1 cells (n=5) (B) react similarly to a 20-second 1 nM resiniferatoxin induction (black bar) with signals that do not return to the base level in the observed time frame. The next two graphs represent capsaicin induction of TRG neurons (n=2) (C) and HT5-1 cells (n=16) (D) in the absence of extracellular calcium. The open bar indicates 60 seconds of induction with 1 μM capsaicin plus 3 mM EGTA, treatment with which does not lead to detectable signals in either cell type. By contrast, the same cells in both cell types responded similarly with a marked elevation of intracellular calcium ion levels when 1 mM calcium is included with a 10-second capsaicin induction (black dot). As indicated in the last two graphs, three iterations of a 10-second long, 330 nM capsaicin induction (black dots) led to tachyphylaxis in both
TRG neurons (n=6) (E) and HT5-1 cells (n=14) (F).

Furthermore, the phenomenon of sustained intracellular calcium ion elevation after moderate capsaicin induction observable in some transiently transfected over-expressing cells [12, 15] is absent in the HT5-1 cell line. The subcellular location of the TRPV1 protein depends on the expression system used. For example, in transiently transfected cos-7 and HEK293 cells, the GFP-labeled TRPV1 protein is located mainly in the endoplasmatic reticulum [15], while in the DRG-derived F-11 cells, it is situated predominantly at the plasma membrane [7]. The location of TRPV1 in intracellular membranes establishes the possibility of calcium ion release from intracellular storage sites upon vanilloid induction, resulting in the elevation of the free calcium ion level in the cytoplasm even in the absence of extracellular calcium [15, 28, 29]. Various expression systems behave differently in this respect, as stably transfected C6 glioma cells do not respond to vanilloid induction without extracellular calcium [12]. Our HT5-1 cell line does not respond to induction with 1 μM capsaicin with intracellular calcium ion level elevation in calcium-free extracellular media, similarly to isolated TRG neurons. This observation concurs well with previous data obtained on sensory neurons [16-19, 30], but contradicts some recent observations where the vanilloid receptor was found on the endoplasmatic reticulum of sensory neurons and was able to release calcium from intracellular stores [26, 31]. The increase in the free calcium ion level in TRPV1-expressing cells induces vesiculation and membrane fragmentation in the mitochondria and the endoplasmatic reticulum, leading to cell death after hours of treatment with vanilloid compounds [3, 15]. Preliminary electron microscopy results show that upon a 30-minute treatment with 1 μM capsaicin, HT5-1 cells show swelling and disruption of the internal structures of the mitochondria, similarly to B-type neurons in the trigeminal ganglia of capsaicin-treated rats [32]. One characteristic of capsaicin-sensitive native sensory neurons is that they exhibit tachyphylaxis resulting in decreased responses upon repeated induction with vanilloids [16-19]. While TRPV1 transfected HEK293 cells behave similarly [3, 13], other TRPV1 expression systems based on stably transfected C6 glioma or CHO cells do not display this important phenomenon [12]. HT5-1 cells display marked tachyphylaxis when repeatedly treated with 330 nM capsaicin, providing a useful model to study the mechanism of TRPV1 desensitization.

In this paper, we reported on the construction of a new stable cell line, HT5-1, expressing an eGFP-tagged version of the rat TRPV1 receptor, which exhibits several properties of vanilloid-sensitive native sensory neurons. The long-term uniform measurable expression of the TRPV1 receptor will make this cell line particularly useful in large-scale pharmacological studies.

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21.6.5 *References*

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