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［総　　説］

化学物質（金属・有機溶剤）の毒性学と産業医としての対応

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要　　旨：わが国でもかつては金属や有機溶剤による産業中毒の事例が多発していた時代があり，このことが機縁の一つとなって1972年（昭和47年）に労働安全衛生法が制定された．現在，化学物質はその危険有害性の程度に応じていくつかの規則によって管理されているが，その対象物質は産業現場で使用される化学物質の一部に過ぎず，毒性が明らかでないまま使用されている化学物質も少なくないのが現状である．労働安全衛生法が改正され，全業種の事業者に化学物質に係るリスクアセスメントが求められるようになっている中で，産業医は毒性が明らかでない化学物質を含めてこれからどのように対応していくべきであろうか．本稿では化学物質の中でも金属と有機溶剤の毒性学に焦点を当てて考察する．

キーワード：有機溶剤，金属，GHS分類，神経毒性，麻酔作用．

1．主な金属の毒性と管理の現状

金属の安全基準について労働安全衛生法では，まず鉛類は鉛中毒予防規則（鉛則），四アルキル鉛中毒予防規則（四アルキル鉛則）により規制されている．わが国の産業中毒としての鉛中毒はかつて蓄電池工場の作業従事者に多く見られていた歴史がある．鉛はタンパク質中のSH基と結合するため非常に有毒であり，多くの研究からヘム生合成反応を触媒する酵素群が最も鉛毒の影響を受けることが明らかとなった．鉛中毒の主症状の一つに貧血があるが，これはヘム生合成過程での最終段階の酵素であるフェロケラターゼの阻害が生じることに起因している．フェロケラターゼ阻害によりその基質である赤血球中プロトポルフィリンが増加することから，これを鉛中毒における生物学的モニタリングの指標の一つにしている．また貧血以外の主症状として下垂手に代表される末梢神経障害があり，ヒトでは軸索変性を主体とする神経変性［1］─動物実験では節性脱髄が主体の神経変性［2］─が報告されている．

四アルキル鉛（労働安全衛生法施行令では，四メチル鉛，四エチル鉛，一メチル・三エチル鉛，二メチル・二エチル鉛および三メチル・一エチル鉛並びにこれを含有するアンチノック剤を四アルキル鉛と呼ぶ）は有機鉛であり，製造工場での事故や輸送中の災害による中毒事例があった．四アルキル鉛は生体内でより毒性の強い三アルキル鉛に変換され，両者ともに脂溶性が高く血液脳関門を容易に通過するため中枢神経症状を生じる．これを反映して四アルキル鉛等業務に常時従事する労働者に対して行われる特殊健康診断では中枢神経症状・精神神経症状の有無の検査が必須となっている．前述した軸索変性のメカニズムを含めて鉛の神経毒性の詳細については未だ不明な点も多いが，これまでの研究から細胞内Ca^{2+}イオンが鉛イオンと置き換わることにより，リン酸化酵素の代表的なものであるプロテインキナーゼCが持続的に活性化されるようになり，細胞内におけるCa^{2+}依存性反応に異常が生じることが神経毒性の一因であると考えられている［3］．

鉛則および四アルキル則が制定された以降は，かつて問題となった高濃度の無機鉛・有機鉛による曝露事例が少なくなってきたことから，特殊健康診断項目についても見直しが進められている．四アルキル鉛業務に常時従事する労働者に対する特殊健康診断の検査項目については平成24年10月より「血液中の鉛の量の検査」および「尿中のデルタアミノレブリン酸の量の検査」が追加され，「血圧の測定」および「好塩基点赤血球数又は尿中のコプロポルフィリンの検査」は除かれるようになった．好塩基点赤血球数はかつて鉛業務の特殊健康診断項目でもあったが，そもそもこれは血

中鉛濃度が80μg/mlを超えないと変動（増加）が認められにくい．今回の法改正によって四アルキル鉛業務の特殊健康診断項目から除外され，血中鉛濃度の検査が加わるようになったことは，四アルキル鉛についても無機鉛と同様により低濃度の曝露に対しての管理が義務付けられるようになったこと意味している．

鉛類以外の金属については特定化学物質障害予防規則（特化則）により規制されている．これまでにベリリウム，水銀，カドミウム，クロム（クロミウム），バナジウム，ニッケル，ヒ素，マンガンといったものがその対象物質であったが，平成25年1月より新たに『インジウム化合物』『コバルト及びその無機化合物』が対象物質に加えられた．インジウムはインジウム・スズ化合物（ITO）として最近は薄型ディスプレイ等の透明電極材料としての需要が高い，いわゆるレアメタルの一つであるが，動物実験において発がん性（国際がん研究機関IARCの区分2A）が認められ，また反復投与毒性としてヒトでは間質性肺炎が認められている．そのため今回定められたインジウム化合物の製造・取扱業務従事者の一次健康診断項目の中には血清インジウム量の測定とともに間質性肺炎のマーカーとされる血清中のシアル化糖鎖抗原KL-6量の測定が含まれている．

コバルトは携帯電話や携帯用音楽プレーヤー，ノートパソコン等に使用されるリチウムイオン2次電池の電極に使用されており，近年その需要が拡大している．主な毒性として発がん性（金属コバルトおよびコバルト化合物はIARC区分2B，コバルト・炭化タングステン合金は区分2A）の他，コバルトはニッケル・クロムとともに金属アレルギーの三大原因金属の一つであることから，アレルギー性接触性皮膚炎や気管支喘息などの恐れもあるため，健診項目には呼吸器症状や皮膚症状の有無の検査が含まれている．

2．主な有機溶剤の毒性と管理の現状

有機溶剤は塗装や洗浄等の産業に広く使用されている．しかしながら揮発性の高いものや脂肪を溶かす作用，すなわち脂溶性が高いものが多く，取扱いを誤ると皮膚や呼吸器を通して容易に体内に吸収され急性中毒や慢性中毒等の健康障害を発生させる恐れがある．有機溶剤中毒の代表的な事例として，1954年（昭和33年）に判明したヘップサンダル製造従事者の骨髄障害がある．ヘップサンダルとは映画「ローマの休日」の中でオードリー・ヘップバーンが履いていたサンダルをヒントに作られたもので，映画の流行とともにこのヘップサンダルの製造が盛んとなった．当時のヘップサンダル製造は下町の家内工業で多くの内職者により行われていたが，サンダルを接着するゴムのりにベンゼン（ベンゾール）が含まれているのを知らずに高濃度のベンゼン蒸気を毎日吸い続けた結果，再生不良性貧血や汎骨髄癆といった骨髄障害（ベンゼン中毒）を生じていたことが判明した．ベンゼンによる骨髄障害の機序については，ベンゼン自身あるいは肝臓におけるベンゼン代謝産物の多能性造血幹細胞・造血前駆細胞や骨髄間質細胞への作用によるものとされているが未だ不明な点も多い[4]．

このベンゼン中毒の発覚と同じ頃に，やはりサンダル（ビニールサンダル）の製造作業従事者で発覚したのがノルマルヘキサン曝露による多発性神経障害である．これもビニールサンダルの製造過程内で使用していたノルマルヘキサン含有の接着剤からの曝露が原因で，家内工業労働者に下肢遠位部の知覚障害に始まる多発神経炎が認められた[5]．このノルマルヘキサン曝露による多発神経炎は代謝産物である2,5-ヘキサンジオンがニューロフィラメント（神経細胞に分布し細胞骨格として機能する）と架橋形成することによるものであることが知られている[6]．これらベンゼン中毒，ノルマルヘキサン中毒を含めて昭和30～40年代はわが国で最も労働災害が多発した時期であり，このような状況を改善すべく1972年（昭和47年）に労働安全衛生法が制定され，特にベンゼン中毒の問題は有機溶剤中毒予防規則（有機則）の機縁となった経緯があるが，現在ベンゼンは特化則の対象物質として，ノルマルヘキサンが有機則の対象物質として管理されている．

有機則および特化則といった特別規則の対象物質でない金属・有機溶剤についても，特定化学物質の環境への排出量の把握等及び管理の改善の促進に関する法律（PRTR法）の対象物質（640物質）であれば，譲渡提供される際にGHS（化学品の分類及び表示に関する世界調和システム）の基づく化学物質安全データシート（SDS）の交付が義務付けられている．2006年（平成18年）4月に施行された改正労働安全衛生法の中で，全業種の事業者に対して化学物質に係るリスクアセスメントの実施，ならびにその結果に基づく健康障害防止措置が努力義務として位置付けられた．産業医もまた人体の仕組みについての専門家として，化学物質がもたらす毒性や健康影響等の判断と評価が今まで以上に求められるようになっており，そのためにもSDSなどを活用して産業現場で使用されている化学物質についての情報を積極的に把握していくことが求められている．

３．リスクアセスメントのための金属の毒性学

わが国の産業界で使用されたことのある又は現に使用されている化学物質は，主なものだけでも約60,000種類，さらに新規化学物質として新たに届け出られ使用されるものは年間1,000種類を超えるといわれているが，当然その全ての物質について毒性に関するデータが十分に提供されているわけではない．化学物質に係るリスクアセスメントが求められる中，産業医としてどのような対応が可能であろうか．

金属については前述したように，いわゆるレアメタルの一つであるインジウムが特化則の対象物質として管理されるようになったばかりである．レアメタルやこの一種であるレアアース（希土類）については需要が増加する一方で，その調達環境の悪化から省・脱レアアース・レアメタル利用部品への代替が推奨され始めていることから，今後産業現場でどの程度の量を扱うようになるかは予測しがたいところもあるが，レアアース・レアメタルを使用する産業現場においては，これらの毒性についても注意しておくことは必要であると思われるが，特にレアアースについての毒性はまだ十分評価されていない．

レアアースの中でもランタノイドには神経毒性に関する報告があり，in vitroの実験からT型電位依存性Ca^{2+}チャネルを抑制する作用が認められることや，抑制性神経伝達を担う$GABA_A$受容体の機能を活性化することなどが報告されている[7]．一方，ラットを用いたランタン（La^{3+}）の経口曝露実験では空間記憶機能に障害が認められている[8]．ほとんどのランタノイドは安定な原子価として3価をとるが，前述したT型Ca^{2+}チャネルの抑制作用の強さはHo^{3+}（ホルミウム・原子番号67）＞Yb^{3+}（イッテルビウム・原子番号70）≧Er^{3+}（エルビウム・原子番号68）＞Gd^{3+}（ガドリニウム・原子番号64）＞Nd^{3+}（ネオジウム・原子番号60）＞Ce^{3+}（セリウム・原子番号58）＞La^{3+}（ランタン・原子番号57）の順であることが報告されている[9]．ランタノイドは原子番号の増加とともに原子半径がわずかずつ小さくなる傾向が見られることが知られていることを考慮すると，この抑制作用はイオンチャネルのチャネル孔のサイズに対するランタノイドイオンの大きさとが関係しているかもしれない．このように金属イオンについては，安定原子価とイオンの大きさから，イオンチャネルに対する作用であれば推定できる可能性があることは興味深い．さらに，心筋に存在するK^+チャネル（HERG）に対してコバルトおよびランタンが抑制作用を示すことがin vitroの実験から報告されているが[10-12]，今のところSDSに心毒性についての記載はない（コバルトについては心臓への影響はあるものの二次的なものとして判断されている）．血液脳関門を通過して初めて生じる中枢神経系のイオンチャネルへの作用と異なり，心筋のイオンチャネルへの作用は化学物質が全身循環に入れば生じる可能性が考えられるので，これらのレアアース・レアメタルの心筋イオンチャネルへの作用，すなわちレアメタル・レアアース曝露と不整脈の発生についてはリスクアセスメントの立場からは注意しておく必要があるのではないだろうか．

４．リスクアセスメントのための有機溶剤の毒性学

有機溶剤についていえば，類似の化学構造を持った化学物質の毒性データを参考にすることも一つの手段となるだろう．大阪の印刷事業場で発生した胆管がんの事例は記憶に新しいところであるが，この原因物質として考えられているのがジクロルメタンおよび1,2-ジクロロプロパンである．ジクロルメタンはすでに有機則の対象物質であり，日本産業衛生学会により1999年（平成11年）に許容濃度が50 ppmと提案されていた．一方，1,2-ジクロロプロパンについては2012年（平成24年）1月28日より化学物質による健康障害防止指針の対象物質の一つに加えられたばかりで，特別規則による規制を受けてはいなかった物質である．ジクロルメタンはハロゲン化された鎖状炭化水素であり，このジクロルメタンと比べ炭素が一つ多い炭化水素骨格を持つものに1,2-ジクロロエタンがある．これはジクロルメタンと比べると沸点が高く（約84℃．ジクロルプロパンは沸点40℃），脂溶性もやや高い．さらにジクロルメタンと同様，すでに有機則の対象物質（第1種有機溶剤．ジクロルメタンは第2種）となっている．この1,2-ジクロロエタンよりさらに炭素が一つ多い炭化水素骨格を持つものが1,2-ジクロロプロパンであり，沸点はさらに高くなって（約96℃）脂溶性も高くなる．沸点が高くなれば揮発性が低くなるので経気道曝露のリスクは低くなるかもしれないが，脂溶性が高くなることで接触した場合の経皮曝露，および体内蓄積のリスクは高くなる可能性が考えられる．もし体内蓄積量が増えれば体内代謝も変わり，代謝産物にも変化が現れるかもしれない．

このように考えていくと，ジクロルメタン，1,2-ジクロロエタンが有機則の対象物質となっていることから，これらの物質よりも沸点は高いものの脂溶性が高くなっている1,2-ジクロロプロパンもまた有機則の対

象物質に相当するリスクを有した有機溶剤の可能性があると，少なくともリスクアセスメントを進める上では考えることが望ましいであろう．現在，ジクロルメタンおよび1,2-ジクロロプロパン曝露による胆管がんの発症の可能性，ならびにそのメカニズムについての解明が進められている．その中でジクロルメタンは高濃度で曝露された場合に，通常のシトクロムP450酵素による代謝経路（CYP経路）が飽和し，代わってグルタチオン-S-転移酵素によるグルタチオン抱合経路（GST経路）による代謝が活性化される．しかもこのグルタチオン-S-転移酵素はヒトでは胆管上皮に多く発現しているため，GST経路による代謝産物がヒトでは胆管がんを発生させるのではないかと考えられている[13]．1,2-ジクロロプロパンの代謝産物の同定やその発がん性の評価が待たれるところではあるが，もしジクロルメタンよりも脂溶性が高い1,2-ジクロロプロパンの方では体内蓄積量が多くなるとすれば，たとえ低濃度であっても長期間の曝露によってCYP経路が飽和しやすくなり，GST経路による代謝が活性化して発がん性を持った代謝産物が産生される，という可能性があるのではないだろうか．

いわゆるシンナー遊びに用いられたトルエンやキシレンに代表されるように，有機溶剤には『吸うと意識が朦朧となる』とイメージしている人も多いだろう．GHS分類にある『健康に対する有害性』の項目には『特定標的臓器毒性（単回曝露）』というのがある[14]．この項目での物質の分類基準として区分1から区分3までの定義があり，区分3の定義（一時的な特定臓器への影響）の最後に『この区分は，麻酔の作用および気道刺激性を含む』と記述されている．つまり中枢神経学的影響という観点からは『麻酔作用』の有無が健康に対する有害性の有無の第一段階になるわけである．さらにこの『麻酔作用』の定義として『眠気，うとうと感，敏捷性の減少，反射の消失，協調の欠如およびめまいといったヒトにおける麻酔作用を含む中枢神経系の抑制を含む．これらの影響は，ひどい頭痛または吐き気としても現れ，判断力低下，めまい，過敏症，倦怠感，記憶機能障害，知覚や協調の欠如，反応時間（の延長）や嗜眠に到ることもある』という記述がある．この定義に記されている症状は，見方を変えれば有機溶剤曝露のない作業従事者にもしばしば認められるような非特異的なものでもある．特に最近問題となっているメンタルヘルス不全でも認められるような症状とも重なるものがあることから，有機溶剤曝露による症状（麻酔作用）であることを見出すには注意が必要であると考えられる．

これまでにトルエン，トリクロロエチレン，1,1,1-トリクロロエタンはいずれも$GABA_A$受容体機能を増強すること[15]，トルエン，四塩化エチレンはいずれも神経型ニコチン性アセチルコリン受容体機能を抑制することが報告されている[16]．これらの受容体は，臨床で用いられる揮発性麻酔薬のような全身麻酔薬の作用部位としても考えられており[17]，前述した化学構造の類似性から推測すると，SDSに記載されていない場合であっても有機溶剤には概ね麻酔作用が出現すると考える方がリスクアセスメントの立場からは妥当であると考えられる．

5．おわりに

GHS分類の導入と改正労働安全衛生法により，金属・有機溶剤を含めて化学物質についてはSDSなどを活用することによって，毒性情報ばかりでなく，沸点や脂溶性といった化学的性質に関する情報までも詳細に提供されるようになり，またそのような情報もインターネットを通じて容易に得られるようになった．毒性に関してはすべてが提供されていない場合もあるので，SDSからの情報だけでは即座に正確な毒性の評価を行うことは難しい．しかしながら，これらの情報を統合して考察することによって，リスクアセスメントの立場からの毒性の推測は可能であると考えられる．金属・有機溶剤の毒性について理解すること，またそれらのリスクアセスメントを実施することに対して，化学物質を化学的にかつ医学的に捉える視点がこれからの産業医に求められるのではないだろうか．

引用文献

1. Buchthal F, Behse F. (1979): Electrophysiology and nerve biopsy in men exposed to lead. Br J Ind Med. 36: 135-147
2. Bouldin TW, Meighan ME, Gaynor JJ, Goines ND, Mushak P, Krigman MR. (1985): Differential vulnerability of mixed and cutaneous nerves in lead neuropathy. J Neuropathol Exp Neurol. 44: 384-396
3. Verity MA. (1990): Comparative observations on inorganic and organic lead neurotoxicity. Environ Health Perspect. 89: 43-48
4. Snyder R. (2012): Leukemia and benzene. Int J Environ Res Public Health. 9: 2875-2893
5. 井上　俊，竹内康浩，竹内寿和子，山田信也，鈴木秀吉，松下敏夫，宮垣仁実，前田勝義，松本忠雄

(1970)：ノルマルヘキサン中毒の多発をみたビニールサンダル業者の労働衛生学的調査研究．産業医学 12: 78-84
6. Agency for Toxic Substances and Disease Registry (ATSDR). (1999): Toxicological profile for n-Hexane. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service
7. Pałasz A, Czekaj P. (2000): Toxicological and cytophysiological aspects of lanthanides action. Acta Biochim Pol. 47: 1107-1114
8. He X, Zhang Z, Zhang H, Zhao Y, Chai Z. (2008): Neurotoxicological evaluation of long-term lanthanum chloride exposure in rats. Toxicol Sci. 103: 354-361
9. Mlinar B, Enyeart JJ. (1993): Block of current through T-type calcium channels by trivalent metal cations and nickel in neural rat and human cells. J Physiol. 469: 639-652
10. Ho WK, Kim I, Lee CO, Youm JB, Lee SH, Earm YE (1999): Blockade of HERG channels expressed in *Xenopus* laevis oocytes by external divalent cations. Biophys J 76: 1959-1971
11. Sanguinetti MC, Jiang C, Curran ME, Keating MT (1995): A mechanistic link between an inherited and an acquired cardiac arrhythmia: HERG encodes the IKr potassium channel. Cell 81: 299-307
12. Sanchez-Chapula JA, Sanguinetti MC. (2000): Altered gating of HERG potassium channels by cobalt and lanthanum. Pflugers Arch. 440: 264-274
13. 井上和也(2012)：「胆管がん」の原因を化学の目で見る－ジクロロメタンの環境リスク論　化学 67: 12-15
14. GHS国連文書　改訂3版：2009年 仮訳http://www.env.go.jp/chemi/ghs/kariyaku3/full.pdf
15. Beckstead MJ, Weiner JL, Eger EI 2nd, Gong DH, Mihic SJ (2000): Glycine and $GABA_A$ receptor function is enhanced by inhaled drugs of abuse. Mol Pharmacol 57: 1199-1205
16. Bale AS, Meacham CA, Benignus VA, Bushnell PJ, Shafer TJ (2005): Volatile organic compounds inhibit human and rat neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. Toxicol Appl Pharmacol 205: 77-88
17. Forman SA, Miller KW. (2011): Anesthetic sites and allosteric mechanisms of action on Cys-loop ligand-gated ion channels. Can J Anaesth. 58: 191-205

上 野　　晋

Toxicology of chemical substances (metals and organic solvents): Management as an occupational physician

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Abstract : Even in Japan, there was a time when cases of occupational poisoning had frequently occurred, which led to the enactment of the Industrial Safety and Health Act in 1972. Currently, the use of only a part of chemical substances utilized in the workplace is regulated according to their designated hazardous level, but there are many other substances whose toxicities have not been elucidated. Risk assessment is now required of entrepreneurs in all categories of industry by the recently-revised Industrial Safety and Health Act. This article will focus on the toxicology of metals and organic solvents, and it will discuss how occupational physicians should manage chemicals, including the ones whose toxicities have not been clarified.

Key words : organic solvents, metals, GHS classification, neurotoxicity, narcotic/anesthetic action.

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• Perspectives and Commentary

Hazardous Metal Pollution in the Republic of Fiji and the Need to Elicit Human Exposure

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The fact that hazardous metals do not bio-degrade or bio-deteriorate translates to long-lasting environmental effects. In the context of evidently rapid global industrialization, this ought to warrant serious caution, particularly in developing countries. In the Republic of Fiji, a developing country in the South Pacific, several different environmental studies over the past 20 years have shown levels of lead, copper, zinc and iron in sediments of the Suva Harbor to be 6.2, 3.9, 3.3 and 2.1 times more than the accepted background reference levels, respectively. High levels of mercury have also been reported in lagoon shellfish. These data inevitably warrant thorough assessment of the waste practices of industries located upstream from the estuaries, but in addition, an exposure and health impact assessment has never been conducted. Relevant government departments are duty-bound, at least to the general public that reside in and consume seafood from the vicinities of the Suva Harbor, to investigate possible human effects of the elevated hazardous metal concentrations found consistently in 20 years of surface sediment analysis. Furthermore, pollution of the intermediate food web with hazardous metals should be investigated, regardless of whether human effects are eventually confirmed present or not.

Keywords Developing countries, Fiji, Hazardous metals, Hazardous metal poisoning, Hazardous metal pollution, Industrialization

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Environmental contamination with hazardous metals should raise concern because metals are not bio-degraded or bio-deteriorated over time like organic pollutants, resulting in long-standing environmental toxicity. In addition to the tendency to bio-accumulate and increase their concentrations over time, hazardous metals are also toxic at low concentrations. These facts ought to raise serious concern in the face of the current trend of global industrialization, particularly in developing countries.

Surface dumping of hazardous metals exposes them to air and rain, thereby generating much acid drainage. Pollution of farmland results in plants absorbing metals and accumulating them in their tissues. This ultimately results in accumulation of these toxins in tissues of animals and marine organisms, and in milk of lactating females [1]. Exposure in human beings results from consumption of contaminated water, plants and animal meat, which induce differential effects on the neurological system and also on human biochemistry. Essentially, all living organisms in any ecosystem are affected at their individual levels on the food chain [1].

We discuss in this paper the situation of hazardous metals in the environment, in the Republic of Fiji (hereafter called "Fiji"),

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a developing country in the South Pacific. Several studies have been conducted in Fiji intermittently over the past 20 years, analyzing environmental levels of a number of hazardous metals. Gangaiya et al. [2] reported in 2001 that copper, lead and zinc concentrations in sediments of certain sites within the Lami estuary were higher than other areas along Suva Harbor, previously considered to be highly contaminated. Maata and Singh [3] documented 7 years later of levels of lead, copper, zinc and iron in sediments of the Suva Harbor to be 6.2, 3.9, 3.3, and 2.1 times more than the accepted background reference levels, respectively. Morrison et al. [4] reported lagoon sediments and shellfish showing high levels of mercury. Chand et al. [5] reported levels of the metalloid arsenic in surficial sediments to be 334 mg/kg in dry weight, almost 30 times more than the USA's Environmental Protection Agency (EPA) cleanup guideline level for sediments of 12 mg/kg, and expected to pose ecological risks.

The history of Fiji shows that its people have primarily made their livelihood by utilizing marine resources within the vicinity of their dwelling areas. However, globalization and economic development over the years has also increased populations and industrialization of urban areas, resulting in over-utilization of marine resources, destruction of habitats, incorrect methods of waste management and depleted marine life [3]. The 2006 Fiji National Liquid Waste Management Strategic Plan reports these changes being significant in the industrial zone of Walu Bay due to high levels of tributyl tin from marine antifouling paints, petroleum pollutants, leached hazardous metals from a battery factory and effluents from nearby food processing factories [6].

Two prime industrial zones are located in the Suva Harbor comprising food-processing factories, a shipyard and large oil storage tanks. Within 3 km away on the coast lie the stinking remains of an open-style, non-segregated rubbish dump that used to accommodate 50,000 tons of all types of waste annually [3]. The dump was decommissioned in 2005 when waste had reached 15 m above ground level. Years of non-segregated, improper waste disposal has caused pesticides, oil and other hazardous pollutants and metals to leach into adjoining waters that flush freshwater into the harbor. Within a km west of this site, is an area of natural beauty that hosts a resort complex and a marina for visiting yachts. People living in the area also use the coastal stretch around this area for recreational purposes and for subsistence fishing [7].

All studies on environmental hazardous metal levels conducted in Fiji to date has been in various areas of the Suva Harbor, located on the south east of the main island. This area serves as a major commercial center with more than 150,000 people, one quarter of the population of the entire main island. Economic growth has resulted in progressive migration of the population from rural to urban centers, associated with a corresponding escalation of environmental problems [3].

Despite all the available scientific data for Fiji confirming elevated levels of several hazardous metals in surficial sediments for the past 20 years, there have been no attempts to establish any effects in human populations, at least in those dwelling in the vicinities of the Suva Harbor. The regular Fijian still lacks a sense of responsibility towards the environment as is evident in the habit of casual trashing and spitting in public spaces, so it may not be overly presumptuous to assume that there is much less awareness of environmental pollution with hazardous metals. It would therefore also be unlikely that there is awareness of neither the bioaccumulation of hazardous metals nor the health effects of human exposure–an area that needs proactive attention and advocacy. Hazardous metal pollution data similar to Fiji's is provided by the South Pacific Regional Environment Program for the neighboring countries of the Solomon Islands, Tahiti, Papua New Guinea and Vanuatu, showing elevated surficial sediment and shellfish concentrations of hazardous metals [8]. Similar to the situation in Fiji, it is also probably unlikely that the general population is aware or informed.

Exposure to hazardous metals is associated with various conditions such as developmental retardation, cancers, kidney failure, autoimmunity, and even death if exposed to very high concentrations [1]. Autoimmunity has been associated with development of diseases of the joint, renal, circulatory and central nervous systems. Lead primarily has neurotoxic effects to which children are more susceptible than adults because they absorb more and excrete less of the lead that their bodies have been exposed to. Relatively more of the retained lead is deposited in the brain, and it causes more damage to the developing brains of children than to mature brains [1].

In a similar manner, the most significant health effect of methylmercury in fetuses, infants, and children is impaired development of the central nervous system. Consumption of methylmercury-contaminated fish and shellfish by a pregnant woman has deleterious effects on the developing fetal neurological system that results in impaired memory, cognition, concentration span, language and fine motor and visuo-spatial skills [1].

There is indeed room for argument in the premises of this paper because it makes reference to hazardous metal levels in surface sediment as the basis for extrapolating a hypothesis of probable human effects. O'Connor and Paulb [9] compared US EPA Environmental Monitoring and Assessment Program Estuaries Program data with bio-effects data on sediment chemistry and toxicity reported by the National Oceanic and Atmospheric Agency, and they could not find any one indicator that accurately predicted toxicity. This finding questions the appropriateness

and reliability of using sediment data as a monitoring tool for contamination and probably deserves more investigation. Eliciting levels of the metabolites and breakdown products of these contaminants may be a valid alternative, as they become the main source of toxicity once the parent compound is no longer detectable. Nevertheless, until an accurate, reliable alternative is developed, surface sediment will continue to provide relevant data on marine environmental health.

It is evident that globalization is pressuring countries into economic development, but industrialization in developing nations like Fiji commonly occurs at the expense of the environment; the release of hazardous metals into the environment should therefore not be expected to reach a plateau anytime soon. Existing environmental data that conclusively prove considerable contamination of the Suva Harbor with various hazardous metals warrant the need for thorough assessment of the waste practices of industries located upstream from the estuaries, but it is also timely that metal contamination of the human food chain and consequent biological effects were investigated. Analyses for hazardous metals in soil, water, dust and where indicated, root crops and vegetables, of proximal residential dwellings need to be considered. Government, non-government and academic institutions with mandates or interest to investigate hazardous metal pollution of the environment should be encouraged to collaborate with the ministry of health and its affiliates in linking this aspect of environmental health to human health. With the effects of climate change already evident in Fiji and its smaller neighboring states, this collaborative proposal provides the added opportunity to address the knowledge gap by raising awareness in the general public about the less-known but vital connections between the environment and human health.

The same proposal is relevant and can be applied to neighboring countries in the Pacific region as well, but for now, the need to conduct exposure and health impact assessments in regards hazardous metals in Fiji, especially in women and children, is pertinent and pressing.

Conflict of Interest

The authors have no conflicts of interest with the material presented in this paper.

References

1. Duruibe JO, Ogwuegbu MC, Egwurugwu JN. Heavy metal pollution and human biotoxic effects. Int J Phys Sci 2007;2(5):112-118.
2. Gangaiya P, Tabudravu J, South R, Sotheeswaran S. Heavy metal contamination of the Lami coastal environment, Fiji. South Pac J Nat Appl Sci 2001;19:(1):24-29.
3. Maata M, Singh S. Heavy metal pollution in Suva harbour sediments, Fiji. Environ Chem Lett 2008;6(2):113-118.
4. Morrison RJ, Gangaiya P, Garimella S, Singh S, Maata M, Chandra A. The future of Suva lagoon. Paper presented at: International Symposium on Science and Management of Suva Lagoon, March 30-April 1, 2005; Suva, Fiji.
5. Chand V, Prasad S, Prasad R. Distribution and chemical fractionation of arsenic in surficial sediments of the Lami coastal environment in Fiji. South Pac J Nat App Sci 2010;28(1):78-81.
6. International Waters of the Pacific Islands. Fiji national liquid waste management strategy and action plan; 2006 [cited 2013 Dec 27]. Available from: http://www.environment.gov.fj/pdf/Waste/Strategies/FInal_Liquid_Waste_Management_Strategy.pdf.
7. Secretariat of the Pacific Regional Environment Programme. Water quality studies on selected South Pacific lagoons. SRS 49 [cited 2013 Jul 10]. Available from: http://www.sprep.org/publications/water-quality-studies-on-selected-south-pacific-lagoons-srs-49.
8. Currey NA, Benko WI. South Pacific regional environment programme marine coastal pollution study (SPREP-POL) [cited 2013 Jul 10]. Available from: http://www.gbrmpa.gov.au/outlook-for-the-reef/great-barrier-reef-outlook-report/outlook-online?sq_content_src=%2BdXJsPWh0dHAlM0ElMkYlMkZ3d3ctcmMuZ2JybXBhLmdvdi5hdSUyRl9fZGF0YSUyRmFzc2V0cyUyRnBkZl9maWxlJTJGMDAxMSUyRjQxNDIlMkZ3czAxNl9wYXBlcl8wOC5wZGYmYWxsPTE%3D.
9. O'Connor TP. Paulb JF, Misfit between sediment toxicity and chemistry. Mar Pollut Bull 2000;40(1):59-64.

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Full Paper

Pentazocine Inhibits Norepinephrine Transporter Function by Reducing its Surface Expression in Bovine Adrenal Medullary Cells

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Abstract. (±)-Pentazocine (PTZ), a non-narcotic analgesic, is used for the clinical management of moderate to severe pain. To study the effect of PTZ on the descending noradrenergic inhibitory system, in the present study we examined the effect of [^{3}H]norepinephrine (NE) uptake by cultured bovine adrenal medullary cells and human neuroblastoma SK-N-SH cells. (−)-PTZ and (+)-PTZ inhibited [^{3}H]NE uptake by adrenal medullary cells in a concentration-dependent (3 – 100 μM) manner. Eadie-Hofstee analysis of [^{3}H]NE uptake showed that both PTZs caused a significant decrease in the V_{max} with little change in the apparent K_m, suggesting non-competitive inhibition. Nor-Binaltorphimine and BD-1047, κ-opioid and σ-receptor antagonists, respectively, did not affect the inhibition of [^{3}H]NE uptake induced by (−)-PTZ and (+)-PTZ, respectively. PTZs suppressed specific [^{3}H]nisoxetine binding to intact SK-N-SH cells, but not directly to the plasma membranes isolated from the bovine adrenal medulla. Scatchard analysis of [^{3}H]nisoxetine binding to SK-N-SH cells revealed that PTZs reduced the B_{max} without changing the apparent K_d. Western blot analysis showed a decrease in biotinylated cell-surface NE transporter (NET) expression after the treatment with (−)-PTZ. These findings suggest that PTZ inhibits the NET function by reducing the amount of NET in the cell surface membranes through an opioid and σ-receptor–independent pathway.

Keywords: adrenal medullary cell, descending noradrenergic inhibitory system, norepinephrine transporter, pentazocine, SK-N-SH cell

Introduction

The racemic compound (±)-pentazocine (PTZ), a non-narcotic analgesic, is used for the management of moderate to severe pain in humans. (−)-PTZ is known to act as an opioid analgesic, and (+)-PTZ is a σ-receptor agonist without analgesic effects. The antinociceptive effects of (−)-PTZ have been reported to be mediated by its agonist action at the κ-opioid receptor (1). Although opioids remain the standard analgesics, there are clinical situations in which alternative approaches to analgesia are desired. For example, physicians are often reluctant to prescribe opioids for a chronic pain condition because of concerns about the potential for abuse and tolerance development. Additionally, opioids are of questionable effectiveness in treating some pain conditions such as neuropathic pain (2).

Analgesia can alternatively be affected through modulation of monoamine activity with serotonin (5-HT) or norepinephrine (NE) uptake inhibitors such as amitriptyline or desipramine (3, 4). These agents are often more effective than opioid analgesics in treating neuropathic pain (5) and are not usually associated with abuse potential. Furthermore, compounds that modify monoamine levels have been reported to modulate opioid-induced analgesia (6 – 8). In some paradigms, the interactions

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between opioid drugs and monoamine uptake inhibitors have been reported as additive, whereas in other models the interactions are synergistic (9, 10).

The NE transporter (NET) is selectively expressed on NE nerve terminals, where it can exert spatial and temporal control over the action of NE (11 – 13). NET induces the termination of neurotransmission by the reuptake of NE released into the extracellular milieu. Human NET, which belongs to the gene family (SLC6A2) of sodium- and chloride-dependent neurotransmitter transporters (12, 14, 15), was the first monoamine transporter to be cloned, and its mRNA is abundantly localized in the brain stem and adrenal medulla (11). NET is also a critical target for various antidepressant and psychostimulants that interact with NET to increase extracellular NE by inhibiting NE uptake (11 – 13).

Adrenal medullary cells derived from the embryonic neural crest share many physiological and pharmacological properties with postganglionic sympathetic neurons. The cells express functional NET proteins (16 – 18). The pharmacological properties of NET in bovine adrenal medullary cells are similar to those of NET in central and peripheral noradrenergic neurons (13). Therefore, NET in bovine adrenal medullary cells has provided a convenient model for studying the effects of various agents such as anesthetics and antipsychotic drugs on this transporter (19, 20). Some centrally acting analgesic agents such as tramadol have both opioid and monoamine modes of action (21, 22). Tramadol inhibits NET function by blocking desipramine-binding sites as the basis for its antinociceptive effect (19). Another opioid analgesic, PTZ, has preliminarily been reported to inhibit the uptake of NE in the rat brain cortex (23), but the precise mechanism remains unclear. In the present study, we investigated the effect of PTZ on NET activity in cultured bovine adrenal medullary cells and SK-N-SH cells and found that PTZ inhibits NET activity through suppression of its cell surface expression in an opioid receptor– and σ-receptor–independent manner.

Materials and Methods

Drugs and reagents were obtained from the following sources: Eagle's minimum essential medium (Eagle's MEM) (Nissui Pharmaceutical, Tokyo); α-MEM, Dulbecco's Modified Eagle's medium (DMEM), l-NE, pargyline hydrochloride, and ascorbic acid (Nacalai Tesque, Kyoto); collagenase (Nitta Zerachin, Osaka); calf serum (Cell Culture Technologies, Gravesano, Switzerland); fetal bovine serum (SAFC Biosciences, Inc., Lenexa, KS, USA); (−)-PTZ, (+)-PTZ, 5-hydroxytryptamine (5-HT), desipramine hydrochloride, clomipramine hydrochloride, naloxone hydrochloride dehydrate, calphostin C, and chelerythrine (Sigma, St. Louis, MO, USA); nisoxetine hydrochloride (Research Biochemicals International, Natick, MA, USA); nor-Binaltorphimine dihydrochloride and GF109203X (Wako, Osaka); BD-1047 dihydrobromide (Tocris Bioscience, Bristol, JK, USA); biotin sucinimidyl ester (sulfo-NHS-biotin), MagnaBind™ Streptavidin Beads (Thermo Scientific, Barrington, IL, USA); L-[7,8-^{3}H]NE, hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)], [*N*-methyl-^{3}H]nisoxetine hydrochloride (Perkin-Elmer Life Sciences, Boston, MA, USA); and $^{45}CaCl_2$ (GE Health Care UK Ltd., Little Chalfont, Buckinghamshire, UK).

Adrenal medullary cells were isolated by collagenase digestion of slices of bovine adrenal medulla. The cells were maintained in a monolayer culture at a density of 1×10^6 cells per well (24-well plate; Corning Life Sciences, Lowell, MA, USA) in culture medium (Eagle's MEM) with 10% calf serum and several antibiotics in 5% CO_2 / 95% air (24). In some experiments, the human noradrenergic neuroblastoma cell line, SK-N-SH (RCB0424), provided by the RIKEN Cell Bank (Tsukuba), was used. SK-N-SH cells were maintained in culture medium containing α-MEM supplemented with 10% fetal bovine serum, and several antibiotics. Cells were plated on poly-L-lysine–coated plates at a density of 0.3×10^6 cells per well in 5% CO_2 / 95% air.

[^{3}H]NE uptake by the cells was performed as follows: Cultured bovine adrenal medullary cells (1×10^6 / well) or SK-N-SH cells (0.3×10^6 / well) were preincubated with or without PTZ for the indicated times and further incubated at 37°C for another 12 min in KRH buffer containing 100 μM pargyline, 1 mM ascorbic acid, and [^{3}H]NE (500 or 100 nM, respectively, 0.1 μCi) in the presence or absence of desipramine and PTZ. KRH buffer was composed of 154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 10 mM HEPES-Tris, and 10 mM glucose, adjusted to pH 7.4. After incubation, the cells were rapidly washed three times with 250 μl of ice-cold KRH buffer and solubilized in 500 μl of 10% Triton X-100. The radioactivity in the solubilized cells was counted with a liquid scintillation counter (Trib-Carb 2900TR; Packard BioScience, Meriden, CT, USA). Desipramine-sensitive uptake was calculated by subtracting the value obtained in the presence of 10 μM desipramine from that obtained in the absence of desipramine (25). In some experiments to determine kinetic parameters, cells were preincubated with or without PTZ (30 μM) for 20 min and then further incubated in the presence of [^{3}H]NE (1 – 30 μM) with or without PTZ for 12 min. The apparent Michaelis constant (K_m) and the maximal velocity (V_{max}) for initial rates of [^{3}H]NE uptake were determined by the Eadie-Hofstee

analysis and calculated by non-linear regression analysis of the data for each individual experiment, using GraphPad Prism 5 software (San Diego, CA, USA).

Specific [^{3}H]nisoxetine binding was proceeded by the following 2 protocols: i) Plasma membranes were prepared from bovine adrenal medulla as described previously (20). The specific binding of [^{3}H]nisoxetine, a selective radioligand for NET, was determined by incubation of membranes (20 μg protein) suspended in a binding buffer (300 mM NaCl, 5 mM KCl, 50 mM Tris-HCl, pH 7.4) for 2 h at 4°C in the presence or absence of 10 μM of nisoxetine. The incubation buffer contained [^{3}H]nisoxetine (2 – 32 nM), and in some experiments additionally PTZ (30 μM). After incubation, binding was terminated by the rapid filtration of the membrane suspension under vacuum through Whatman GF/C glass fiber filters. Specific binding of [^{3}H]nisoxetine was defined as the binding inhibited by the selective NET inhibitor nisoxetine (10 μM). ii) For [^{3}H]nisoxetine binding assays in intact cells, SK-N-SH cells were treated with or without PTZ at 37°C for 30 min and were incubated in 0.3 ml of ice-cold binding buffer (100 mM NaCl, 50 mM Tris, 100 μM ascorbic acid, pH 8.0) containing PTZ and [^{3}H]nisoxetine (2 – 64 nM) at 4°C for 2 h. After washing the cells, the radioactivity in the solubilized cells was counted. Non-specific binding was determined in the presence of 10 μM nisoxetine and the specific binding was obtained by subtracting non-specific binding from the total binding.

Cell surface biotinylation assay was proceeded as described previously (26) with a slight modification. After incubation of SK-N-SH cells with or without (−)-PTZ (100 μM) for 30 min at 37°C, biotinylation was carried out by incubating the cells for 1 h at 4°C in 250 μl of phosphate-buffered saline (PBS)/Ca^{2+}/Mg^{2+} containing 1.5 mg/ml of sulfo-NHS-biotin. The biotinylation reagent was removed, and cells were washed twice with PBS/Ca^{2+}/Mg^{2+} containing 100 mM glycine, followed by quenching for 30 min with the same reagent and three times washed with PBS/Ca^{2+}/Mg^{2+}. Cells in each well were solubilized by gentle shaking for 1 h in 200 μl of radioimmunoprecipitation (RIPA) buffer containing protease inhibitors (Nacalai Tesque). Cell lysates were centrifuged at 20,000 × *g* for 30 min, and an aliquot of each sample was used for the isolation of biotinylated proteins with streptavidin beads by incubating for 1 h at room temperature with gentle shaking. The biotinylated proteins were then washed five times with RIPA buffer and then eluted by heating the beads in sample buffer at 95°C for 5 min.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as follows: The NET proteins in total, nonbiotinylated and biotinylated fractions were separated by SDS-PAGE (10%), and were electroblotted onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P) with transfer buffer (39 mM glycine, 48 mM Tris, 0.0375% SDS, 20% methanol, pH 8.5). After blocking with a blocking buffer (PVDF Blocking Reagent for Can Get Signal; Toyobo, Osaka) for 1 h at room temperature, the membranes were incubated with a primary antibody against NET (1:1,000; Santa Cruz Biotechnology, Calfornia, USA) or β-actin (1:10,000; Cell Signaling Technology, Beverly, MA, USA) in Can Get Signal Solution-1 (Toyobo) for 1 h at room temperature and then washed with Tris-buffered saline-Tween (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) (TBS-T). The immunoreactive bands were reacted in a solution (Can Get Signal Solution-2; Toyobo) with a polyclonal goat anti-rabbit antibody conjugated to horseradish peroxidase (1:10,000; Cell Signaling Technology) for 1 h at room temperature, and washed repeatedly as above. The immunoreactive bands were visualized by Immobilon Western (Millipore Corporation, Billerica, MA, USA) and quantified by Light-Capture with the CS Analyzer (ATTO Corporation, Tokyo).

COS-7 cells (RCB0539; RIKEN Cell Bank) were maintained in culture medium containing DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rat serotonin transporter (rSERT) cDNA was used as previously reported (27). Transfection of rSERT cDNA was performed using Effectene Transfection Reagent (Qiagen, Hilden, Germany) at 10:1 (reagent:cDNA) ratios. Cells were incubated after transfection for 24 to 48 h before use in the experiments. Cells (1 × 10^6 / well) were preincubated at 37°C for 20 min in KRH buffer in the presence or absence of PTZ. The cells were further incubated with KRH buffer containing 10 μM pargyline, 100 μM ascorbic acid, and [^{3}H]5-HT (50 nM, 0.1 μCi) at 37°C for 12 min in the presence or absence of PTZ. Nonspecific uptake was determined in the presence of 10 μM clomipramine.

The influx of $^{45}Ca^{2+}$ was measured in cultured bovine adrenal medullary cells as reported previously (24). Cells (4 × 10^6 per dish) were incubated with 1.5 μCi of $^{45}CaCl_2$ at 37°C for 5 min with or without 56 mM K^+ and PTZ in KRH buffer. After incubation, the cells were washed 3 times with ice-cold KRH buffer, solubilized in 10% Triton X-100, and the radioactivity counted.

All experiments were performed in duplicate or triplicate, and each experiment was repeated at least three times. All values are given as means ± S.E.M. Data were statistically evaluated by Student's *t*-test or one-way analysis of valiance (ANOVA). If a significant F value was found, Dunnett's test for multiple compari-

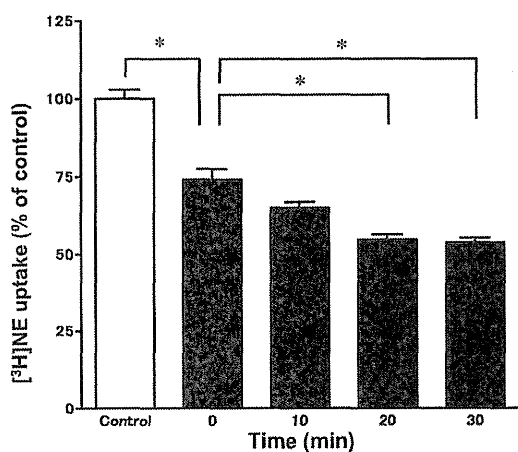


Fig. 1. Time course of effect of (−)-PTZ on [^{3}H]NE uptake in cultured bovine adrenal medullary cells. After preincubation with or without (−)-PTZ (30 μM) for the indicated period, the cells were incubated with or without (−)-PTZ (30 μM) at 37°C for another 12 min in the presence of [^{3}H]NE (500 nM). The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control in which cells were not treated with (−)-PTZ (30 μM) during all procedures. The values of the control [^{3}H]NE uptake was 0.23 ± 0.02 pmol / 10^6 cells per min. $*P < 0.05$, compared with the control or 0 min.

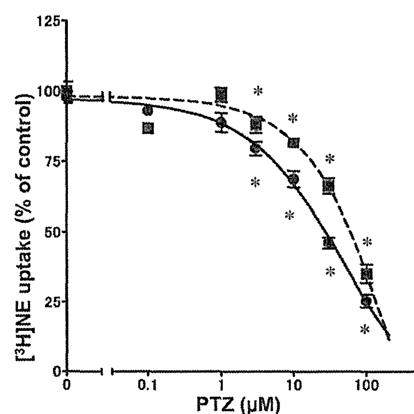


Fig. 2. Concentration–response curves of effects of (−)- and (+)-PTZ on [^{3}H]NE uptake by cultured bovine adrenal medullary cells. After preincubation with (−)-PTZ (closed circles) or (+)-PTZ (closed squares) for 20 min, cells were incubated with 500 nM [^{3}H]NE and various concentrations (0.1 – 100 μM) of PTZ for 12 min at 37°C. The desipramine- sensitive [^{3}H]NE uptake by the cells was measured. Data were expressed as a percentage of the control (0.38 ± 0.08 pmol / 10^6 cells per min). $*P < 0.05$, compared with 0 μM PTZ.

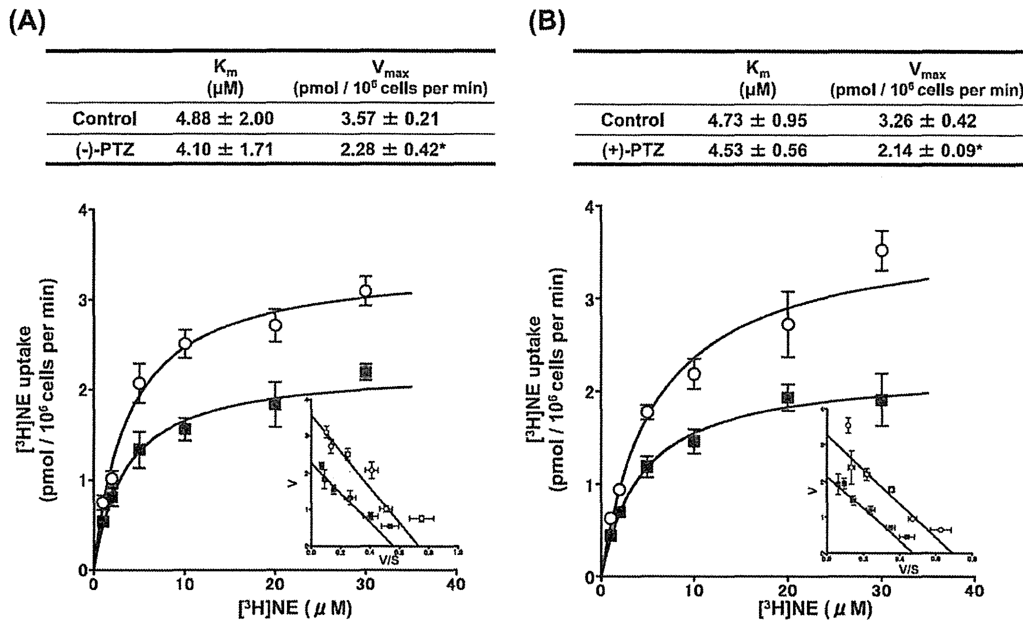
sons was carried out to identify differences among groups. When $P < 0.05$, the differences were considered statistically significant.

Results

(−)-PTZ (30 μM) inhibited [^{3}H]NE uptake by cultured bovine adrenal medullary cells in a time-dependent manner (Fig. 1). Preincubation of cells with (−)-PTZ (30 μM) caused a decrease in [^{3}H]NE uptake by the cells for up to 30 min, with a continuously maximal reduced level occurring at 20 min. Therefore, evaluation of PTZ's effect on [^{3}H]NE uptake was performed using cells pretreated with PTZ for 20 min. Treatment with (−)- or (+)-PTZ (3 – 100 μM) significantly inhibited [^{3}H]NE uptake in a concentration-dependent manner (Fig. 2). The half-maximal inhibitory concentrations (IC_{50}) for inhibition of [^{3}H]NE uptake by (−)- and (+)- PTZ were calculated as 54.59 ± 2.89 μM and 72.38 ± 2.96 μM, respectively. Incubation of cells with increasing concentrations of [^{3}H]NE (1 – 30 μM) showed that [^{3}H]NE uptake was saturable in both control and PTZ-treated cells (Fig. 3). Eadie-Hofstee analysis showed that both (−)- and (+)-PTZ caused a significant decrease in the maximal velocity (V_{max}) of [^{3}H]NE uptake with little change in the apparent Michaelis constant (K_m).

To investigate the involvement of opioid receptors and σ-receptors, we used naloxone, nor-Binaltorphimine, and BD-1047 as a non-selective opioid receptor antagonist, selective κ-opioid receptor antagonist, and selective σ-receptor antagonist, respectively. No antagonists, however, altered the effects of either (−)-PTZ, a κ-opioid receptor agonist, or (+)-PTZ, a σ-receptor agonist, on [^{3}H]NE uptake (Fig. 4), suggesting an opioid receptor– and σ-receptor–independent pathway. Since it is well-known that activation of protein kinase C down-regulates NET function, we examined the involvement of protein kinases in [^{3}H]NE uptake reduced by PTZ. Inhibitors of protein kinase C (calphostin C, chelerythrine, and GF109203X) did not affect the PTZ-induced inhibition of [^{3}H]NE uptake (Fig. 5). Furthermore, H-89, an inhibitor of cAMP-dependent protein kinase, and wortmannin, a phosphoinositide 3-kinase inhibitor, also had little effect (data not shown).

To determine the site of PTZ's action on NET, we examined the effects of PTZ on the specific binding of [^{3}H]nisoxetine, an specific inhibitor of NET, to plasma membranes isolated from bovine adrenal medulla. The specific binding of [^{3}H]nisoxetine to plasma membranes was saturable with an increasing concentration of 2 – 32 nM [^{3}H]nisoxetine, although (−)- and (+)-PTZ (30 μM) did not inhibit [^{3}H]nisoxetine binding (Fig. 6). Scatchard plot analysis showed that (−)- and (+)-PTZ (30 μM) had little effect on the maximal binding (B_{max}) or the dissociation constant (K_d) in comparison with that of the control. Since we could not observe the saturation curve of [^{3}H] nisoxetine specific binding to intact bovine adrenal



(A)

	K_m (μM)	V_{max} (pmol / 10^6 cells per min)
Control	4.88 ± 2.00	3.57 ± 0.21
(-)-PTZ	4.10 ± 1.71	2.28 ± 0.42*

(B)

	K_m (μM)	V_{max} (pmol / 10^6 cells per min)
Control	4.73 ± 0.95	3.26 ± 0.42
(+)-PTZ	4.53 ± 0.56	2.14 ± 0.09*

Fig. 3. Saturation curve of [^{3}H]NE uptake and its Eadie-Hofstee analysis. After preincubation with (closed squares) or without (open circles) (−)-PTZ (A) and (+)-PTZ (B) (30 μM) for 20 min, cells were incubated with various concentrations (1 – 30 μM) of [^{3}H]NE in the presence (closed squares) or absence (open circles) of (−)-PTZ (A) and (+)-PTZ (B) (30 μM) for 12 min at 37°C. [^{3}H]NE uptake was measured. Lower inset: Eadie-Hofstee analysis of [^{3}H]NE uptake. Data are means ± S.E.M. of three separate experiments carried out in duplicate. V, velocity (pmol / 10^6 cells per min); V/S, velocity / substrate concentration (pmol/10^6 cells·min·μM). *$P < 0.05$, compared with the control.

medullary cells, we used SK-N-SH cells, a human noradrenergic neuroblastoma cell line, instead of cultured adrenal medullary cells. Both (−)- and (+)-PTZ suppressed the specific binding of [^{3}H]nisoxetine (2 – 64 nM) to SK-N-SH cells and reduced the B_{max} of [^{3}H]nisoxetine binding without any change in the K_d (Fig. 7). (−)-PTZ (1.0 – 100 μM) suppressed the specific binding of [^{3}H]nisoxetine to SK-N-SH cells in a concentration-dependent manner (Fig. 8A) similar to that of [^{3}H]NE uptake by the cells (Fig. 8B). To examine whether (−)-PTZ–induced inhibition of [^{3}H]NE uptake or [^{3}H]nisoxetine binding occurs as a result of changes in surface expression of NETs, we determined the effect of (−)-PTZ on the population of NET proteins accessible to the membrane impermeant biotinylation reagent in human neuroblastoma SK-N-SH cells (Fig. 9). (−)-PTZ caused a significant decrease in the ratio of density of NET band in biotinylated fractions to that of total fraction to 52.2% of the control (Fig. 9B) and to β-actin to 61.7% of the control (Fig. 9C).

To investigate whether the inhibitory effect of PTZ on the transport function is specific for NET or not, we examined the effect of PTZ on another transporter, serotonin transporter (SERT), in rSERT cDNA transfected COS-7 cells. As shown in Fig. 10, (−)- and (+)-PTZ caused a significant reduction in [^{3}H]5-HT uptake in a concentration-dependent manner. We further checked the effect of PTZ on another membrane protein, voltage-dependent Ca^{2+} channels, by measuring $^{45}Ca^{2+}$ influx after treatment of cells with PTZ. (−)- and (+)-PTZ (30 μM), however, had little effect on 56 mM K^+–induced $^{45}Ca^{2+}$ influx (data not shown), suggesting that PTZ preferentially inhibits the function of monoamine transporters.

Discussion

PTZ, a non-narcotic analgesic with weak narcotic antagonistic activity, is advocated for the relief of moderate to severe pain. (−)-PTZ is a κ-opioid receptor agonist that induces analgesic effects, whereas (+)-PTZ is a σ-receptor agonist without analgesic effects. In addition to these receptors, we examined the effect of PTZ on NET function in adrenal medullary cells and SK-N-SH cells, a noradrenergic neuroblastoma cell line, to search for the active site of PTZ's effect on the descending noradrenergic pain modulatory pathways. In the present study, (−)-PTZ and (+)-PTZ significantly inhibited [^{3}H]NE uptake in a concentration (3.0 – 100 μM)-dependent manner. The inhibitory effect of (+)-PTZ (IC_{50} = 72.4 μM) was slightly less potent than that of (−)-PTZ

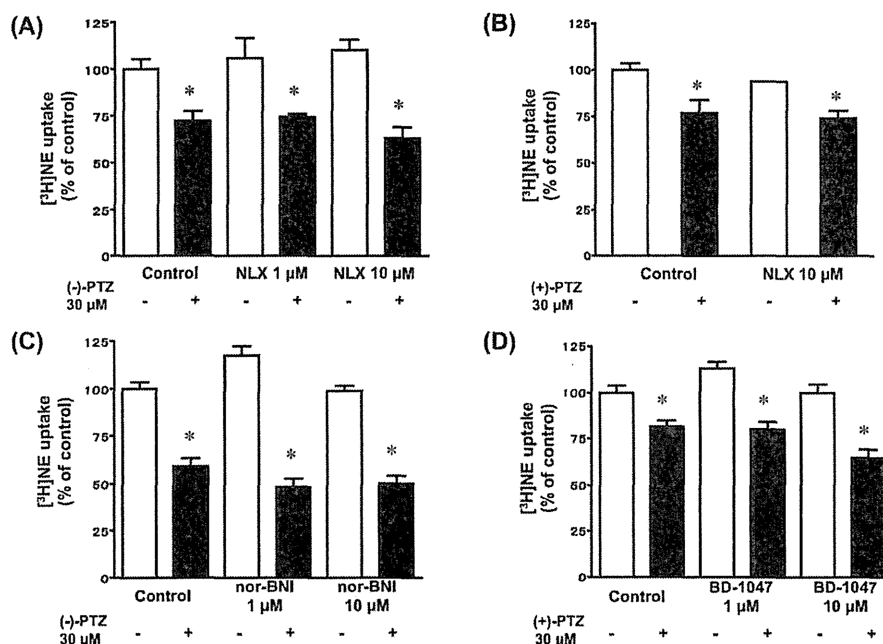


Fig. 4. Effect of naloxone (A and B), nor-Binaltorphimine (C), and BD-1047 (D) on (−)- or (+)-PTZ–induced inhibition of [^{3}H]NE uptake. A and B) After preincubation with or without the nonselective opioid receptor antagonist naloxone (NLX, 1 or 10 μM) for 20 min, cells were incubated for 12 min with [^{3}H]NE in the presence (black column) or absence (white column) of (−)-PTZ (A) or (+)-PTZ (B) (30 μM) and naloxone (NLX). C and D) After preincubation with or without the κ-opioid receptor antagonist nor-Binaltorphimine (nor-BNI, 1 or 10 μM) (C) or the σ-receptor antagonist BD-1047 (1 or 10 μM) (D) for 20 min, cells were incubated for 12 min with [^{3}H]NE in the presence (black column) or absence (white column) of (−)-PTZ (C) or (+)-PTZ (D) (30 μM) and each antagonist. The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control. *$P < 0.05$, compared with each 0 μM PTZ.

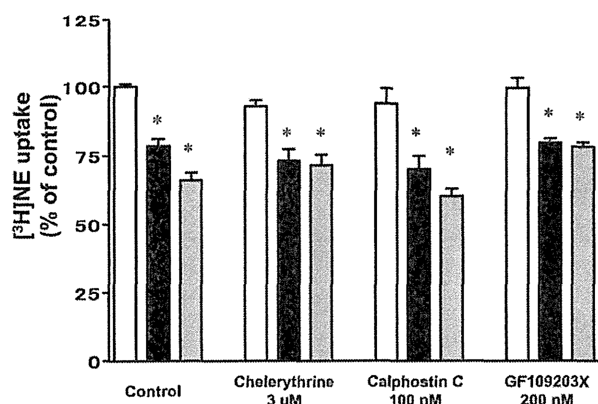


Fig. 5. Effect of various inhibitors of protein kinase C on PTZ-induced inhibition of [^{3}H]NE uptake. The cells were pretreated for 20 min with or without various inhibitors of protein kinase C and then incubated for 12 min with [^{3}H]NE in the presence (black column or gray column) or absence (white column) of (−)-PTZ (black column) or (+)-PTZ (gray column) (30 μM) and various inhibitors of protein kinase C. The desipramine-sensitive [^{3}H]NE uptake by the cells were measured. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control. *$P < 0.05$, compared with each 0 μM PTZ.

(IC_{50} = 54.6 μM). As much as 80% – 90% of NE released from presynaptic terminals is believed to be physiologically taken up again by the presynaptic neurons, thereby terminating neurotransmission (12). Therefore, even a slight inhibition of NET activity induced by PTZ may enhance noradrenergic neurotransmission.

Down-regulation of NET function by PTZ

To study the site(s) of action of PTZ on NET, we examined the effects of PTZ on kinetic parameters for [^{3}H]NE uptake by the cells. The Eadie-Hofstee analysis of [^{3}H]NE uptake revealed that PTZ induces a decrease in the V_{max} of [^{3}H]NE uptake without any change in the K_m. These results suggest that PTZ inhibits the NET function by interacting with a site or sites other than the recognition site for NE. To investigate the possible involvement of opioid receptors, we used naloxone, a nonselective opioid receptor antagonist; nor-Binaltorphimine, a selective κ-opioid receptor antagonist; and BD-1047, a selective σ-receptor antagonist. None of these opioid and σ-receptor antagonists reversed the suppression of [^{3}H]NE uptake induced by (−)- and (+)-PTZ, suggesting that these effects are independent of opioid and σ receptors.

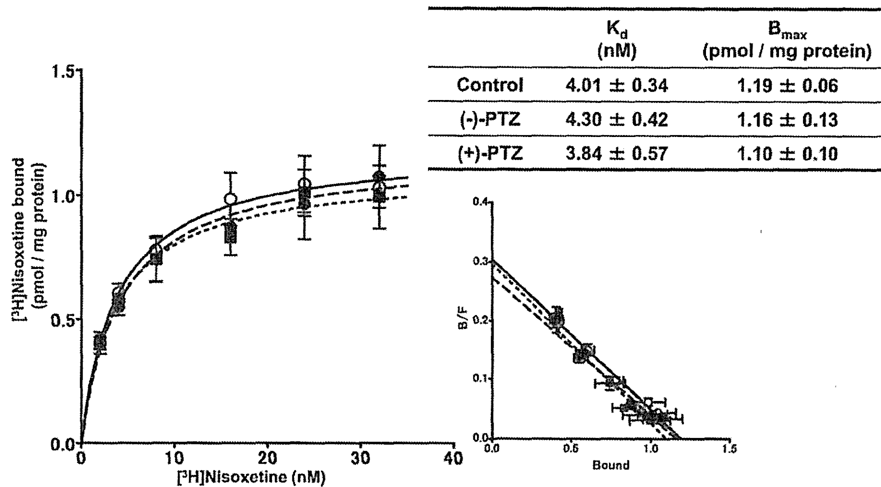


Fig. 6. Effects of (−)- or (+)-PTZ on specific binding of [^{3}H]nisoxetine to plasma membranes of bovine adrenal medulla and its Scatchard plot analysis. Plasma membranes isolated from bovine adrenal medulla were incubated at 4°C for 2 h with (closed circles or closed squares) or without (open circles) (−)-PTZ (closed circles) or (+)-PTZ (closed squares) (30 μM) in the presence of increasing concentrations (2 – 32 nM) of [^{3}H]nisoxetine. The specific binding of [^{3}H]nisoxetine was measured. Right inset: Scatchard plot analysis data of [^{3}H]nisoxetine binding. Data are means ± S.E.M. of three separate experiments carried out in duplicate. B, bound (pmol / mg protein); B/F, bound/free (pmol / mg protein per nM).

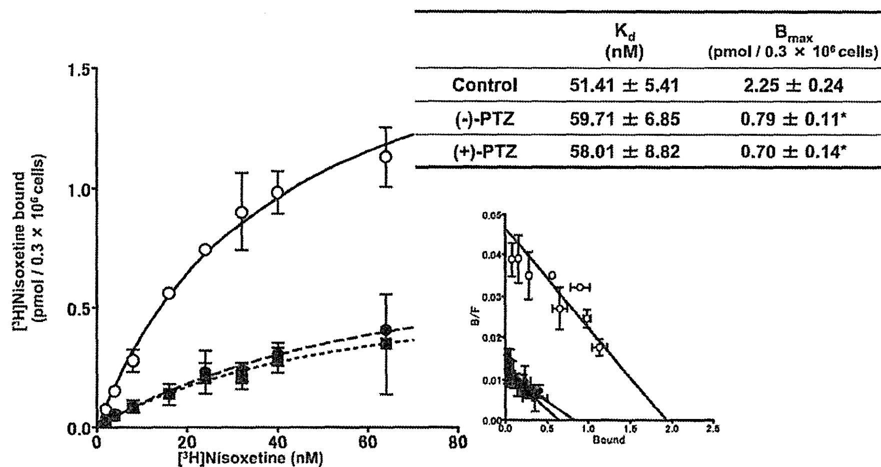


Fig. 7. Effects of (−)- or (+)-PTZ on specific binding of [^{3}H]nisoxetine to intact SK-N-SH cells and its Scatchard plot analysis. After preincubation with (closed circles or closed squares) or without (open circles) (−)-PTZ (closed circles) or (+)-PTZ (closed squares) at 30 μM, SK-N-SH cells (0.3 × 10^6 cells) were incubated in the presence of increasing concentrations (2 – 64 nM) of [^{3}H] nisoxetine with or without (−)- or (+)-PTZ (30 μM) at 4°C for 2 h. The specific binding of [^{3}H]nisoxetine was measured. Right inset: Scatchard plot analysis data of [^{3}H]nisoxetine binding. Data are means ± S.E.M. of three separate experiments carried out in duplicate. B, bound (pmol / 0.3 × 10^6 cells); B/F, bound/free (pmol / 0.3 × 10^6 cells per nM). *$P < 0.05$, compared with the control.

NET is regulated by a number of intracellular signaling pathways. One common pathway is phosphorylation by several protein kinases such as cAMP-dependent protein kinase and protein kinase C (15, 28). Activation of protein kinase C is reported to down-regulate the function of NET in SK-N-SH cells (29) or transfected COS-7 cells (30). In the present study, inhibitors of protein kinase C (calphostin C, chelerythrine, and GF109203X), cAMP-dependent protein kinase (H-89), and phosphoinositide 3-kinase (wortmannin) had little effect on PTZ-induced inhibition of [^{3}H]NE uptake, suggesting that these protein kinases are not involved in the PTZ-

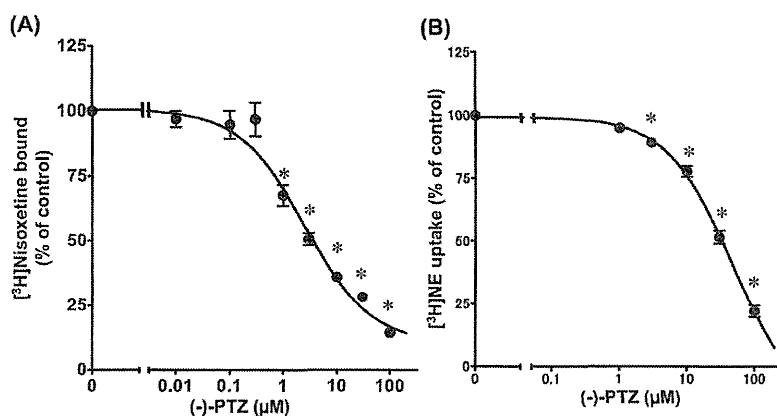


Fig. 8. Effect of various concentrations of (−)-PTZ on [^{3}H]nisoxetine specific binding (A) and [^{3}H]NE uptake (B) by SK-N-SH cells. A) After preincubation with various concentrations of (−)-PTZ for 30 min, the cells were incubated in [^{3}H]nisoxetine at 4°C for 2 h. The specific binding of [^{3}H]nisoxetine (20 nM) was measured. Data are means ± S.E.M. of three separate experiments carried out in duplicate and expressed as % of the control. The values of the control (0 μM PTZ) were 0.52 ± 0.01 pmol / 0.3×10^6cells. B) After preincubation with various concentrations of (−)-PTZ for 20 min, cells were incubated in the presence of [^{3}H]NE (100 nM) with various concentrations of (−)-PTZ for 12 min. The desipramine-sensitive [^{3}H]NE uptake by the cells was measured. Data were expressed as a percentage of the control (0.45 ± 0.04 pmol / 10^6 cells per min). $^*P < 0.05$, compared with 0 μM PTZ.

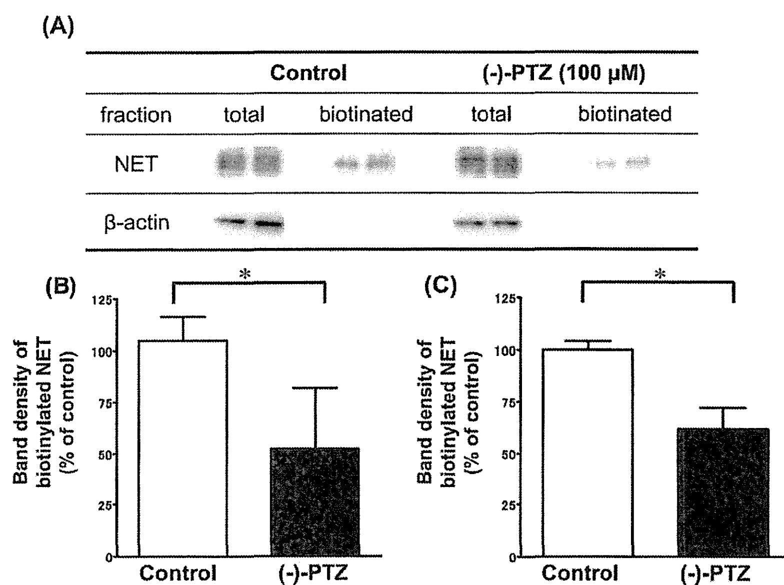


Fig. 9. Effect of (−)-PTZ on cell surface expression of NET proteins in SK-N-SH cells. SK-N-SH cells were pretreated with (black column) or without (white column) (−)-PTZ (100 μM) for 30 min and then biotinylated with sulfo-NHS-biotin. A) Aliquots of total and nonbiotinylated fractions were loaded, whereas the entire eluate from streptavidin beads was loaded as the biotinylated sample and blots were probed with NET antibody as described. B, C) Data are means ± S.E.M. of three separate experiments and are expressed as % of the control NET band [the ratio of the density of biotinylated NET fraction (54 kDa) to that of total NET] (B) and as % of the control (the ratio of the density of biotinylated NET fraction to that of β-actin) (C). $^*P < 0.05$, compared with the control.

induced down-regulation of NET function.

Treatment of cells with PTZ for 30 min caused a suppression in the specific binding of [^{3}H]nisoxetine to intact SK-N-SH cells, although PTZ did not directly inhibit the specific binding of [^{3}H]nisoxetine to plasma membranes isolated from bovine adrenal medulla. Scatchard plot analysis showed that PTZ significantly decreased the B_{max} with little change in the K_d in SK-N-SH cells. These findings suggest that treatment of cells with PTZ inhibits the specific binding of [^{3}H]nisoxetine by reducing the binding sites of [^{3}H]nisoxetine on NET. Indeed, in the present study, we observed the decrease in membrane surface expression of NET proteins after treatment with PTZ. At present, however, the intracellular mechanism of PTZ-induced down-regulation of NET remains to be clarified. This is probably due to a reduction of the membrane trafficking of NET to the plasma membrane or an increase in its degradation or endocytosis by the lysozomal degradation system. The latter possibility may partially be excluded by the sub-

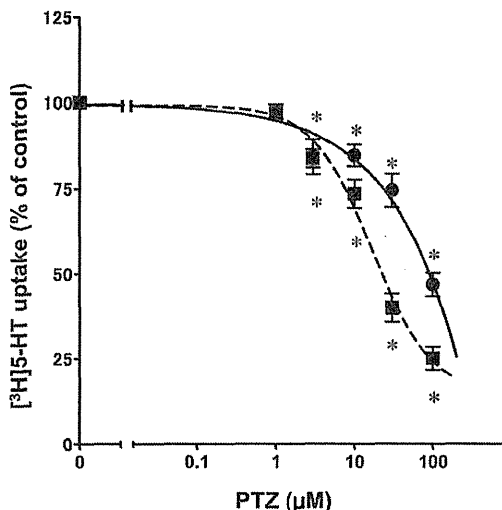


Fig. 10. Effects of (−)- and (+)-PTZ on [^{3}H]5-HT uptake by rat SERT cDNA transfected COS-7 cells. After transfection with cDNA rSERT for 24 – 48 h, COS-7 cells (1 × 10^6 / well) were preincubated at 37°C for 20 min in KRH buffer in the presence or absence of various concentrations of (−) (closed circles)- or (+) (closed squares)-PTZ. The cells were further incubated with [^{3}H]5-HT (50 nM, 0.1 μCi) at 37°C for 12 min in the presence or absence of (−)- or (+)-PTZ. Data are means ± S.E.M. of three separate experiments carried out in triplicate and expressed as % of the control (0.43 ± 0.01 pmol / 10^6 cells per min). $^*P < 0.05$, compared with 0 μM each PTZ.

sequent finding that bafilomycin A1, a lysosomal inhibitor, did not reverse the inhibition of [^{3}H]NE uptake induced by PTZ (data not shown). Further study will be required to determine the mechanism by which PTZ down-regulates NET function.

The pharmacological significance of PTZ-induced down-regulation of NET

After intramuscular administration of 40 or 80 mg of PTZ, mean peaks of PTZ plasma concentration at 15 min were 102 and 227 ng/ml (0.318 and 0.707 μM), respectively (31). In the present study, the [^{3}H]NE uptake in adrenal medullary cells was significantly inhibited by PTZ at 3.0 – 100 μM, and the specific binding of [^{3}H] nisoxetine and [^{3}H]NE uptake of intact SK-N-SH cells were significantly inhibited by PTZ at 1 – 100 μM and 3.0 – 100 μM, respectively. Taken together, the present findings and previous data suggest that near-clinical concentrations of PTZ partly suppress the NET function of cultured bovine adrenal medullary cells.

Several lines of evidence have shown that the descending inhibitory system consists of noradrenergic and/or serotonergic neurons (32, 33). A recent study reported a potential use of 5-HT_7 receptor agonists as adjuvants of opioid analgesia because spinal activation of 5-HT_7 receptors has a role in the expression of opiate-induced analgesia through activation of descending inhibition (34). Furthermore, the antinociceptive effects of some clinical drugs, such as tricyclic antidepressants, are partially explained by enhanced noradrenergic or serotonergic neurotransmission induced by suppression of the NET or SERT in the descending inhibitory system in the brain and spinal cord (35). Indeed, in the present study, PTZ inhibited not only NET function but also SERT function, suggesting a preferential inhibition by PTZ of monoamine transporter functions. Furthermore, several anesthetics such as ketamine and propofol also inhibited the NET function (36, 37). Taken together with these results, it is intriguing to propose that PTZ induces antinociceptive effects via the down-regulation of NET and/or SERT in addition to the activation of opioid receptors.

Readers assessing the significance of the present findings should bear in mind the limitations of this study. First, the cellular mechanism by which PTZ induces the down-regulation of NET function has not been elucidated. Further investigations, including those on NET membrane trafficking and internalization or degradation, are needed to clarify its molecular mechanism after exposure to PTZ. Second, although cultured bovine adrenal medullary cells or SK-N-SH cells are a good in vitro model system of noradrenergic neurons, in vivo animal studies of PTZ are required to establish the involvement of NET down-regulation by PTZ in its antinociceptive effect.

In conclusion, the present findings suggest that near-clinical concentrations of PTZ induce the down-regulation of NET via suppression of cell surface expression of NET proteins. This may add a new antinociceptive aspect of PTZ to our pharmacological understanding of analgesics.

Acknowledgments

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References

1 Gutstein HB, Akil H. Opioid analgesics. In: Hardman JG, Limbird LE, Goodman-Gilman A, editors. Goodman & Gilman's the pharmacological basis of therapeutics. 10th ed. New York: McGraw-Hill; 2001. p. 569–619.
2 McQuay HJ. Pharmacological treatment of neuralgic and neuropathic pain. Cancer Surv. 1988;7:141–159.

3 Sharav Y, Singer E, Schmidt E, Dionne RA, Dubner R. The analgesic effect of amitriptyline on chronic facial pain. Pain. 1987;31:199–209.
4 Magni G. The use of antidepressants in the treatment of chronic pain. A review of the current evidence. Drugs. 1991;42: 730–748.
5 Max MB, Lynch SA, Muir J, Shoaf SE, Smoller B, Dubner R. Effects of desipramine, amitriptyline, and fluoxetine on pain in diabetic neuropathy. N Engl J Med. 1992;326:1250–1256.
6 Larson AA, Takemori AE. Effect of fluoxetine hydrochloride (Lilly 110140), a specific inhibitor of serotonin uptake, on morphine analgesia and the development of tolerance. Life Sci. 1977;21:1807–1811.
7 Malseed RT, Goldstein FJ. Enhancement of morphine analgesia by tricyclic antidepressants. Neuropharmacology. 1979;18:827–829.
8 Ossipov MH, Malseed RT, Goldstein FJ. Augmentation of central and peripheral morphine analgesia by desipramine. Arch Int Pharmacodyn Ther. 1982;259:222–229.
9 Ossipov MH, Harris S, Lloyd P, Messineo E. An isobolographic analysis of the antinociceptive effect of systemically and intrathecally administered combinations of clonidine and opiates. J Pharmacol Exp Ther. 1990;255:1107–1116.
10 Bergman SA, Wynn RL, Alvarez L, Asher K, Thut PD. Imipramine-fentanyl antinociception in a rabbit tooth pulp model. Life Sci. 1991;49:1279–1288.
11 Pacholczyk T, Blakely RD, Amara SG. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature. 1991;350:350–354.
12 Amara SG, Kuhar MJ. Neurotransmitter transporters: recent progress. Annu Rev Neurosci. 1993;16:73–93.
13 Bönisch H, Brüss M. The noradrenaline transporter of the neuronal plasma membrane. Ann N Y Acad Sci. 1994;733: 193–202.
14 Baker EL, Blakely RD. Norepinephrine and serotonin transporters. In: Bloom FE, Kupfer DJ, editors. Psychopharmacology. 4th ed. New York: Raven Press; 1995. p. 321–333.
15 Zahniser NR, Doolen S. Chronic and acute regulation of Na^+/Cl^--dependent neurotransmitter transporters: drugs, substrates, presynaptic receptors, and signaling systems. Pharmacol Ther. 2001;92:21–55.
16 Michael-Hepp J, Blum B, Bönisch H. Characterization of the [^{3}H]-desipramine binding site of the bovine adrenomedullary plasma membrane. Naunyn-Schmiedeberg's Arch Pharmacol. 1992;346:203–207.
17 Ceña V, García AG, Montiel C, Sánchez-García P. Uptake of [^{3}H]-nicotine and [^{3}H]-noradrenaline by cultured chromaffin cells. Br J Pharmacol. 1984;81:119–123.
18 Lingen B, Brüss M, Bönisch H. Cloning and expression of the bovine sodium- and chloride-dependent noradrenaline transporter. FEBS Lett. 1994;342:235–238.
19 Sagata K, Minami K, Yanagihara N, Shiraishi M, Toyohira Y, Ueno S, et al. Tramadol inhibits norepinephrine transporter function at desipramine-binding sites in cultured bovine adrenal medullary cells. Anesth Analg. 2002;94:901–906.
20 Hara K, Yanagihara N, Minami K, Ueno S, Toyohira Y, Sata T, et al. Ketamine interacts with the noradrenaline transporter at a site partly overlapping the desipramine binding site. Naunyn Schmiedeberg's Arch Pharmacol. 1998;358:328–333.
21 Raffa RB, Friderichs E, Reimann W, Shank RP, Codd EE, Vaught JL. Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic. J Pharmacol Exp Ther. 1992;260:275–285.
22 Driessen B, Reimann W. Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain in vitro. Br J Pharmacol. 1992;105:147–151.
23 Kinouchi K, Maeda S, Saito K, Inoki R, Fukumitsu K, Yoshiya I. Effects of d- and l-pentazocine on the release and uptake of norepinephrine in rat brain cortex. Res Commun Chem Pathol Pharmacol. 1989;63:201–213.
24 Yanagihara N, Oishi Y, Yamamoto H, Tsutsui M, Kondoh J, Sugiura T, et al. Phosphorylation of chromogranin A and catecholamine secretion stimulated by elevation of intracellular Ca^{2+} in cultured bovine adrenal medullary cells. J Biol Chem. 1996;271:17463–17468.
25 Toyohira Y, Yanagihara N, Minami K, Ueno S, Uezono Y, Tachikawa E, et al. Down-regulation of the noradrenaline transporter by interferon-α in cultured bovine adrenal medullary cells. J Neurochem. 1998;70:1441–1447.
26 Kippenberger AG, Palmer DJ, Comer AM, Lipski J, Burton LD, Christie DL. Localization of the noradrenaline transporter in rat adrenal medulla and PC12 cells: evidence for its association with secretory granules in PC12 cells. J Neurochem. 1999;73:1024–1032.
27 Morikawa O, Sakai N, Obara H, Saito N. Effects of interferon-α, interferon-γ and cAMP on the transcriptional regulation of the serotonin transporter. Eur J Pharmacol. 1998;349:317–324.
28 Mandela P, Ordway GA. The norepinephrine transporter and its regulation. J Neurochem. 2006;97:310–333.
29 Apparsundaram S, Galli A, DeFelice LJ, Hartzell HC, Blakely RD. Acute regulation of norepinephrine transport: I. protein kinase C-linked muscarinic receptors influence transport capacity and transporter density in SK-N-SH cells. J Pharmacol Exp Ther. 1998;287:733–743.
30 Bönisch H, Hammermann R, Brüss M. Role of protein kinase C and second messengers in regulation of the norepinephrine transporter. Adv Pharmacol. 1998;42:183–186.
31 Yeh SY, Todd GD, Johnson RE, Gorodetzky CW, Lange WR. The pharmacokinetics of pentazocine and tripelennamine. Clin Pharmacol Ther. 1986;39:669–676.
32 Pertovaara A. Noradrenergic pain modulation. Prog Neurobiol. 2006;80:53–83.
33 Fields HL, Basbaum AI. Central nervous system mechanisms of pain modulation. In: Wall PD, Melzack R, editors. Textbook of Pain. 4th ed. Edinburgh: Churchill Livingstone; 1999. p. 309–329.
34 Brenchat A, Ejarque M, Zamanillo D, Vela JM, Romero L. Potentiation of morphine analgesia by adjuvant activation of 5-HT_7 receptors. J Pharmacol Sci. 2011;116:388–391.
35 Monks R, Merskey H. Psychotropic drugs. In: Wall PD, Melzack R, editors. Textbook of pain. 4th ed. Edinburgh: Churchill Livingstone; 1999. p. 1155–1186.
36 Hara K, Yanagihara N, Minami K, Hirano H, Sata T, Shigematsu A, et al. Dual effects of intravenous anesthetics on the function of norepinephrine transporters. Anesthesiology. 2000;93:1329–1335.
37 Minami K, Yanagihara N, Segawa K, Tsutsui M, Shigematsu A, Izumi F. Inhibitory effects of propofol on catecholamine secretion and uptake in cultured bovine adrenal medullary cells. Naunyn-Schmiedeberg's Arch Pharmacol. 1996;353:572–578.

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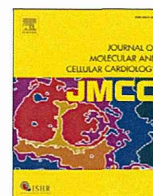

Original article

Development of an experimentally useful model of acute myocardial infarction: 2/3 nephrectomized triple nitric oxide synthases-deficient mouse

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A B S T R A C T

We investigated the effect of subtotal nephrectomy on the incidence of acute myocardial infarction (AMI) in mice deficient in all three nitric oxide synthases (NOSs). Two-thirds nephrectomy (NX) was performed on male triple NOSs$^{-/-}$ mice. The 2/3NX caused sudden cardiac death due to AMI in the triple NOSs$^{-/-}$ mice as early as 4 months after the surgery. The 2/3NX triple NOSs$^{-/-}$ mice exhibited electrocardiographic ST-segment elevation, reduced heart rate variability, echocardiographic regional wall motion abnormality, and accelerated coronary arteriosclerotic lesion formation. Cardiovascular risk factors (hypertension, hypercholesterolemia, and hyperglycemia), an increased number of circulating bone marrow-derived vascular smooth muscle cell (VSMC) progenitor cells (a pro-arteriosclerotic factor), and cardiac up-regulation of stromal cell-derived factor (SDF)-1α (a chemotactic factor of the progenitor cells) were noted in the 2/3NX triple NOSs$^{-/-}$ mice and were associated with significant increases in plasma angiotensin II levels (a marker of renin–angiotensin system activation) and urinary 8-isoprostane levels (a marker of oxidative stress). Importantly, combined treatment with a clinical dosage of an angiotensin II type 1 receptor blocker, irbesartan, and a calcium channel antagonist, amlodipine, markedly prevented coronary arteriosclerotic lesion formation and the incidence of AMI and improved the prognosis of those mice, along with ameliorating all those pro-arteriosclerotic parameters. The 2/3NX triple NOSs$^{-/-}$ mouse is a new experimentally useful model of AMI. Renin–angiotensin system activation, oxidative stress, cardiovascular risk factors, and SDF-1α-induced recruitment of bone marrow-derived VSMC progenitor cells appear to be involved in the pathogenesis of AMI in this model.

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1. Introduction

Acute myocardial infarction is a disorder in which cardiac myocytes undergo necrosis as a consequence of interrupted coronary blood flow [1]. Acute myocardial infarction is a major cause of morbidity and mortality worldwide, with more than 7 million people in the world suffering from acute myocardial infarction each year [1]. Over the past two decades, the in-hospital mortality rate after admission for acute myocardial infarction has substantially declined to less than 10%, owing to

Abbreviations: ACE, angiotensin-converting enzyme; ADMA, asymmetric dimethylarginine; AMI, acute myocardial infarction; APC, activated protein C; apo E, apolipoprotein E; AT_1, angiotensin II type 1; CKD, chronic kidney disease; ECG, electrocardiography; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; mAb, monoclonal antibody; NO, nitric oxide; NOS, NO synthase; NX, nephrectomy; Sca-1^{+}, stem cell antigen-1^{+}; SDF-1α, stromal cell-derived factor-1α; VSMC, vascular smooth muscle cell; WHHL, Watanabe heritable hyperlipidemic; WT, wild-type.

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