

Fig. 2. Effects of raloxifene (A, C) or tamoxifen (B, D) on basal (A, B) and ACh (C, D)-induced ¹⁴C-catecholamine synthesis from [¹⁴C]tyrosine in the cells. Cells (4×10^6 / dish) were incubated with L-[U-¹⁴C]tyrosine (20 μ M, 1 μ Ci) and various concentrations of raloxifene (RLX) (A, C) or tamoxifen (TAM) (B, D) at 37°C for 20 min in the presence (C, D) or absence (A, B) of ACh (300 μ M). ¹⁴C-Labelled catechol compounds were separated by ion exchange chromatography on Duolite C-25 columns (H⁺ type, 0.4 \times 7.0 cm) and counted for radioactivity. Control ¹⁴C-catecholamine synthesis [$20,500 \pm 3,900$ (A) and $20,800 \pm 6,500$ (B) dpm / 4×10^6 cells / 20 min] and ACh-induced synthesis [$50,600 \pm 3,900$ (C) and $112,000 \pm 8,000$ (D) dpm / 4×10^6 cells / 20 min] were assigned a value of 100% and the data are expressed as % of control or ACh. Values shown are the mean \pm S.E.M. of 4 experiments carried out in duplicate. Data are expressed as the mean \pm S.E.M. of 4 experiments carried out in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; compared with each control. † $P < 0.05$ and †† $P < 0.01$; compared with ACh alone.

Effects of raloxifene on ACh responses in *Xenopus* oocytes expressing nAChRs

The direct effect of raloxifene on ACh responses in *Xenopus* oocytes expressing rat $\alpha 4\beta 2$ nAChRs was examined. As shown in Fig. 8, raloxifene (1.0 μ M) reversibly inhibited ACh-induced Na⁺ currents.

Discussion

In the present study, we demonstrated the stimulatory or inhibitory effects of two SERMs, raloxifene and tamoxifen, on specific [³H]17 β -E₂ binding to plasma membrane estrogen receptors as well as catecholamine synthesis and secretion in bovine adrenal medullary cells.

Raloxifene and tamoxifen are allosteric modulators of plasma membrane estrogen receptors

SERMs are well-known to bind to estrogen-binding sites of classical nuclear ERs to initiate changes in formation on the ER, the dissociation of the ER from heat-shock proteins, and various gene transcriptions (2). In the present study, raloxifene at 1.0–10 nM and tamoxifen at 1.0 nM–10 μ M except for 100 nM rather enhanced [³H]17 β -E₂ binding to plasma membrane estrogen receptors, whereas raloxifene at higher concentrations (1.0–10 μ M) inhibited it. This finding suggests that raloxifene and tamoxifen are an allosteric modulator of membrane estrogen receptors and that raloxifene at higher concentrations interferes with the specific binding of [³H]17 β -E₂ to membrane estrogen receptors. The

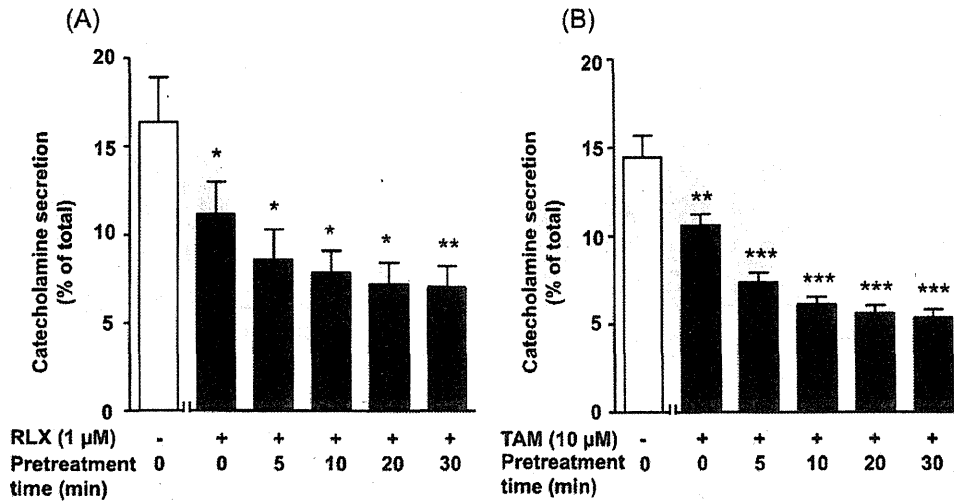


Fig. 3. Time course of pretreatment effect of raloxifene and tamoxifen on ACh-induced catecholamine secretion from the cell. After preincubation with (closed column) or without (open column) 1 μ M raloxifene (RLX) (A) or 10 μ M tamoxifen (TAM) (B) for the indicated period, the cells (10^6 /well) were stimulated with ACh (300 μ M) for 10 min at 37°C. Catecholamines secreted into the medium were measured and expressed as a percentage of the total catecholamines [$7.19 \pm 0.98 \mu$ g (A), $7.60 \pm 0.82 \mu$ g (B)] in the cells. Data are the mean \pm S.E.M. of 4 separate experiments carried out in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; compared with ACh alone.

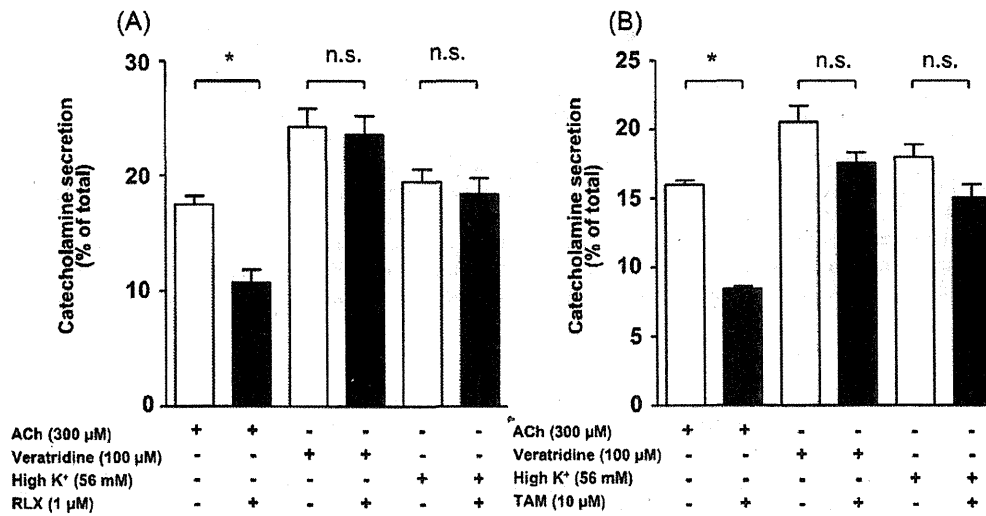


Fig. 4. Effects of raloxifene (A) or tamoxifen (B) on catecholamine secretion induced by various secretagogues. After preincubation of cells with or without raloxifene (RLX) (A) or tamoxifen (TAM) (B) for 10 min, the cells (10^6 /well) were incubated with or without ACh (300 μ M), veratridine (100 μ M), or high concentrations of K⁺ (56 mM) for another 10 min at 37°C. Catecholamines secreted into the medium were measured and expressed as a percentage of the total catecholamines [$6.21 \pm 1.19 \mu$ g (A), $7.28 \pm 0.59 \mu$ g (B)] in the cells. Data are the mean \pm S.E.M. of 4 separate experiments carried out in triplicate. * $P < 0.05$, compared with ACh alone.

former result is similar to that of our previous data produced by ICI182,780, a pure antagonist of nuclear ER, and *p*-nonylphenol or bisphenol A, environmental

estrogenic pollutants, both of which allosterically enhanced specific [³H]17 β -E₂ binding to plasma membrane estrogen receptors (12).

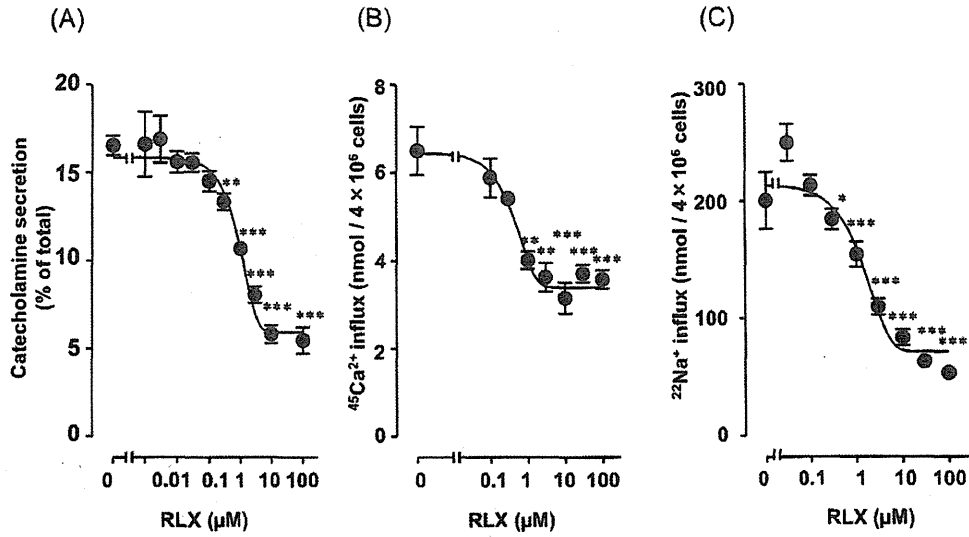


Fig. 5. Effects of various concentrations of raloxifene on ACh-induced catecholamine secretion (A), $^{45}\text{Ca}^{2+}$ influx (B), and $^{22}\text{Na}^{+}$ influx (C) in the cells. A) After preincubation of cells with various concentrations of raloxifene (RLX) for 10 min at 37°C , cells (10^6 /well) were stimulated with ACh ($300\ \mu\text{M}$) in the presence of various concentrations of raloxifene for another 10 min at 37°C . Catecholamines secreted were measured and expressed as a percentage of total catecholamines ($5.01 \pm 0.37\ \mu\text{g}$). B and C) After preincubation with various concentrations of raloxifene for 10 min, cells (4×10^6 / dish) were incubated in the presence of various concentrations of raloxifene, $300\ \mu\text{M}$ ACh, $1.5\ \mu\text{Ci}$ of $^{45}\text{CaCl}_2$ (B), or $^{22}\text{NaCl}$ (C) for another 5 min at 37°C . $^{45}\text{Ca}^{2+}$ influx and $^{22}\text{Na}^{+}$ influx were measured, and expressed as $\text{nmol} / 4 \times 10^6$ cells. Data are the mean \pm S.E.M. of 4 separate experiments carried out in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; compared to ACh alone.

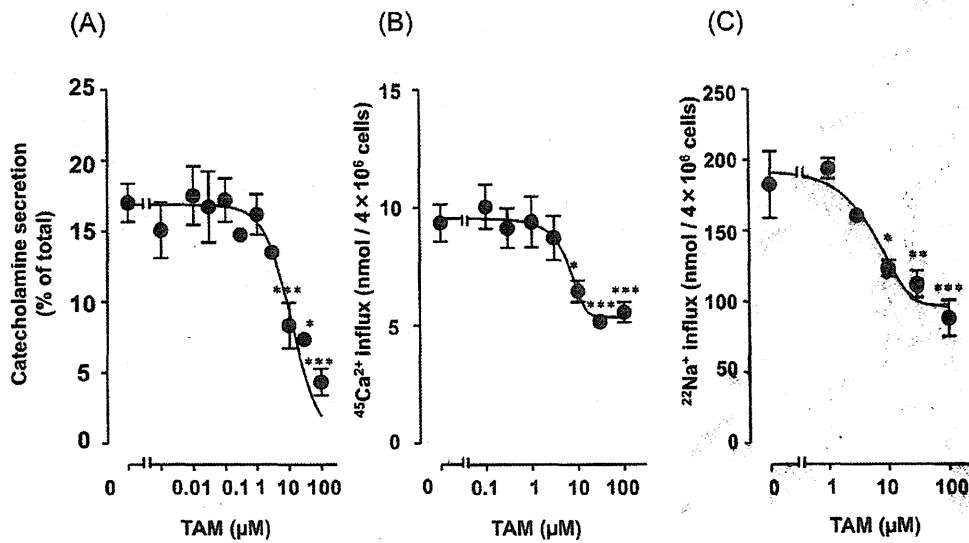


Fig. 6. Effects of various concentrations of tamoxifen on ACh-induced catecholamine secretion (A), $^{45}\text{Ca}^{2+}$ influx (B), and $^{22}\text{Na}^{+}$ influx (C) in the cells. A) After preincubation of cells with various concentrations of tamoxifen (TAM) for 10 min at 37°C , cells (10^6 /well) were stimulated with ACh ($300\ \mu\text{M}$) in the presence of various concentrations of tamoxifen for another 10 min at 37°C . Catecholamines secreted were measured and expressed as a percentage of total catecholamines ($5.64 \pm 0.49\ \mu\text{g}$). B and C) After preincubation with various concentrations of tamoxifen for 10 min, the cells (4×10^6 / dish) were incubated in the presence of various concentrations of tamoxifen, $300\ \mu\text{M}$ ACh, $1.5\ \mu\text{Ci}$ of $^{45}\text{CaCl}_2$ (B), or $^{22}\text{NaCl}$ (C) for another 5 min at 37°C . $^{45}\text{Ca}^{2+}$ influx and $^{22}\text{Na}^{+}$ influx were measured, and expressed as $\text{nmol} / 4 \times 10^6$ cells. Data are the mean \pm S.E.M. of 4 separate experiments carried out in triplicate. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$; compared to ACh alone.

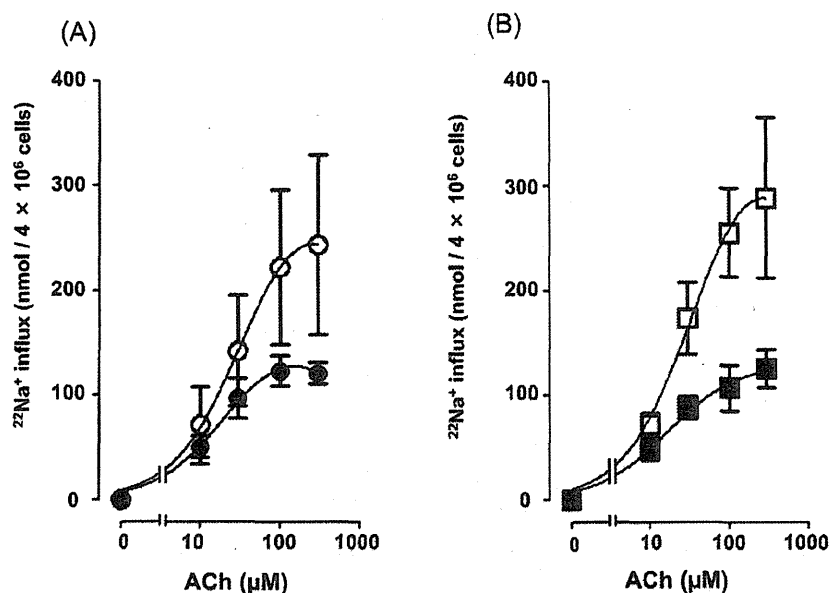


Fig. 7. Inhibitory mode of raloxifene (A) and tamoxifen (B) on $^{22}\text{Na}^+$ influx induced by ACh. After preincubation of cells with or without raloxifene (1.0 μM) and tamoxifen (10 μM) for 10 min, the cells were incubated with (closed circle) or without (open circle) raloxifene (1.0 μM) (A) and with (closed square) or without (open square) tamoxifen (10 μM) (B) in the presence of 1.5 μCi of $^{22}\text{NaCl}$ and ACh (3–300 μM) for 5 min at 37°C. $^{22}\text{Na}^+$ influx was measured and expressed as nmol / 4×10^6 cells. Data are the mean \pm S.E.M. from 3 separate experiments carried out in triplicate.

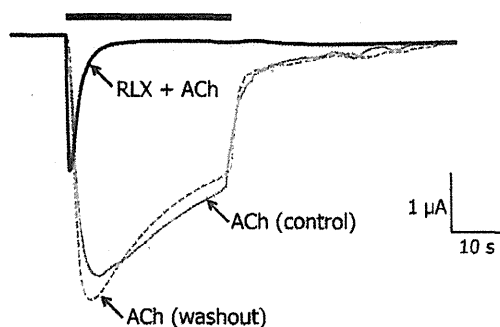


Fig. 8. Effect of raloxifene on ACh-induced response in nAChRs expressed in *Xenopus* oocytes. Representative current tracings obtained from the same *Xenopus* oocyte expressing rat $\alpha 4\beta 2$ nAChRs were superimposed, demonstrating an inhibitory effect of raloxifene (RLX, 1 μM) on the current induced by the EC_{50} of ACh. Traces represent the responses of ACh (control), in the presence of RLX (RLX + ACh), and 15-min washout [ACh (washout): dotted line]. The bar indicates the time of drug applications (ACh alone or RLX plus ACh), but it should be noted that RLX was pre-applied for 2 min before the coapplication with ACh.

Modulatory effects of raloxifene and tamoxifen on ^{14}C -catecholamine synthesis and catecholamine secretion

In bovine adrenal medullary cells, we previously reported that $17\beta\text{-E}_2$ (12), environmental estrogenic pollutants (19), and phytoestrogens, such as daidzein (13) and resveratrol (14), stimulate catecholamine synthesis through plasma membrane estrogen receptors. A previous study reported that activation of membrane

estrogen receptors increases intracellular Ca^{2+} concentrations and progesterone synthesis in rat hypothalamic astrocytes (20). In the present study, however, the stimulatory effect of tamoxifen on ^{14}C -catecholamine synthesis may not be mediated through the plasma membrane estrogen receptors because it increased basal synthesis of ^{14}C -catecholamines only at 100 nM. Furthermore, at higher concentrations raloxifene (0.1–1.0 μM) and tamoxifen (1.0–10 μM) inhibited ^{14}C -catecholamine synthesis. There was no relation between catecholamine synthesis and modulation of $[\text{^3H}]17\beta\text{-E}_2$ binding induced by SERMs. From these present results, it seems that the interactions of the SERMs with plasma membrane estrogen receptors are not associated with catecholamine synthesis in the cells.

Both raloxifene (1.0 μM) and tamoxifen (10–100 μM) suppressed ACh-induced ^{14}C -catecholamine synthesis in a concentration-dependent manner. We previously reported that ACh activates nAChR-ion channels, thereby inducing Na^+ influx and then Ca^{2+} influx as well as catecholamine synthesis (10) and secretion (11). In the present study, raloxifene and tamoxifen both preferentially inhibited catecholamine secretion mediated through nAChRs but neither did so through voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. The present results were partially consistent with those of a previous report (7) that raloxifene at micromolar concentrations inhibited catecholamine output elicited by ACh or high potassium in perfused rat adrenal glands and cultured bovine adrenal medullary cells. In the present study, both SERMs suppressed ACh-induced $^{45}\text{Ca}^{2+}$

influx and $^{22}\text{Na}^+$ influx in a concentration-dependent manner, similar to their suppression of catecholamine secretion and synthesis. It is likely that raloxifene and tamoxifen suppress ACh-induced catecholamine synthesis and secretion primarily by inhibiting Na^+ influx through nAChR-ion channels and subsequent Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

We further investigated the inhibitory mechanisms underlying the effects of raloxifene and tamoxifen on nAChR-ion channels. Increased concentrations of ACh did not overcome the inhibitory effects of the SERMs on ACh-induced $^{22}\text{Na}^+$ influx, suggesting that the SERMs act on the sites differently than they act on ACh binding sites of nAChR-ion channels. We confirmed that raloxifene directly and reversibly suppressed ACh-induced Na^+ current in *Xenopus* oocytes expressing rat $\alpha 4\beta 2$ nAChR.

Pharmacological significance of the effects of the SERMs on catecholamine synthesis and secretion

The pharmacokinetic properties of raloxifene and tamoxifen in postmenopausal women and in women with breast cancer showed that the maximum plasma concentrations of raloxifene and tamoxifen were 2–3 nM and 20–330 nM, respectively, during clinical treatments (21, 22). Tamoxifen, however, is reported to accumulate in tissues, resulting in 100-fold higher concentrations than in plasma after repeated administration of the drug during long-term treatment for breast cancer (23). Therefore, the concentrations of raloxifene and tamoxifen used in the present study should be high, compared to those plasma therapeutic concentrations, but they might be clinically relevant in the tissues.

Several lines of evidence have shown that the SERMs have both potentially adverse (24) and beneficial effects on brain functions such as cognition (25, 26) and neuroprotection (27). Indeed, raloxifene is reported to induce neurite outgrowth in estrogen receptor-positive PC12 cells (28). In the present study, we demonstrated that low concentration of tamoxifen (100 nM) stimulates basal catecholamine synthesis, whereas at high concentrations, it inhibits basal and ACh-induced catecholamine synthesis and secretion. On the basis of the present results, it would be hypothesized that the SERMs negatively or positively modulate the functions of central noradrenergic or dopaminergic neurons, depending on their concentrations, by changing the synthesis and release of each neurotransmitter. To confirm this possibility, further *in vivo* studies are required in the near future.

In summary, we demonstrated that two SERMs, raloxifene and tamoxifen, allosterically interact with plasma membrane estrogen receptors, whereas at high

concentrations each of them inhibits catecholamine synthesis and secretion induced by ACh in adrenal medullary cells and probably in peripheral and central sympathetic neurons.

Acknowledgments

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Conflicts of Interest

The authors have no conflict of interest to report.

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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、全業種の事業者には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む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Ca²⁺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Ca²⁺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80 $\mu\text{g}/\text{ml}$ 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)として最近で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Ca²⁺チャン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(La³⁺)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Ca²⁺チャンネルの抑制作用の強さはHo³⁺(ホルミウム・原子番号67) > Yb³⁺(イッテルビウム・原子番号70) ≥ Er³⁺(エルビウム・原子番号68) > Gd³⁺(ガドリニウム・原子番号64) > Nd³⁺(ネオジウム・原子番号60) > Ce³⁺(セリウム・原子番号58) > La³⁺(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ながら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ンネルのチャンネル孔のサイズに対するランタノイドイオンの大きさ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、イオンチャン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⁺チャンネル(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ンネルへの作用と異なり、心筋のイオンチャン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ンネルへの作用、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ロメタンおよび1,2-ジクロロプロパンである。ジクロロ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。ジクロロメタンはハロゲン化された鎖状炭化水素であり、このジクロロ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。これはジクロロメタンと比べると沸点が高く(約84℃、ジクロロプロパンは沸点40℃)、脂溶性もやや高い、さらにジクロロメタンと同様、すでに有機則の対象物質(第1種有機溶剤、ジクロロメタンは第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、ジクロロメタン、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、麻酔的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。

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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

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 farm-

land 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.

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Full Paper

Pentazocine Inhibits Norepinephrine Transporter Function by Reducing its Surface Expression in Bovine Adrenal Medullary CellsGo Obara^{1,2}, Yumiko Toyohira², Hirohide Inagaki², Keita Takahashi², Takafumi Horishita¹, Takashi Kawasaki¹, Susumu Ueno³, Masato Tsutsui⁴, Takeyoshi Sata¹, and Nobuyuki Yanagihara^{2,*}¹Department of Anesthesiology, School of Medicine, ²Department of Pharmacology, School of Medicine,³Department of Occupational Toxicology, Institute of Industrial Ecological Sciences,

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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 [³H]norepinephrine (NE) uptake by cultured bovine adrenal medullary cells and human neuroblastoma SK-N-SH cells. (–)-PTZ and (+)-PTZ inhibited [³H]NE uptake by adrenal medullary cells in a concentration-dependent (3–100 μM) manner. Eadie-Hofstee analysis of [³H]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 [³H]NE uptake induced by (–)-PTZ and (+)-PTZ, respectively. PTZs suppressed specific [³H]nisoxetine binding to intact SK-N-SH cells, but not directly to the plasma membranes isolated from the bovine adrenal medulla. Scatchard analysis of [³H]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), 1-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 succinimidyl ester (sulfo-NHS-biotin), MagnaBind™ Streptavidin Beads (Thermo Scientific, Barrington, IL, USA); L-[7,8-³H]NE, hydroxytryptamine creatinine sulfate, 5-[1,2-³H(N)], [N-methyl-³H]nisoxetine hydrochloride (Perkin-Elmer Life Sciences, Boston, MA, USA); and ⁴⁵CaCl₂ (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% CO₂ / 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% CO₂ / 95% air.

[³H]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 μ M pargyline, 1 mM ascorbic acid, and [³H]NE (500 or 100 nM, respectively, 0.1 μ Ci) in the presence or absence of desipramine and PTZ. KRH buffer was composed of 154 mM NaCl, 5.6 mM KCl, 1.1 mM MgSO₄, 2.2 mM CaCl₂, 10 mM HEPES-Tris, and 10 mM glucose, adjusted to pH 7.4. After incubation, the cells were rapidly washed three times with 250 μ l of ice-cold KRH buffer and solubilized in 500 μ l of 10% Triton X-100. The radioactivity in the solubilized cells was counted with a liquid scintillation counter (Tri-Carb 2900TR; Packard BioScience, Meriden, CT, USA). Desipramine-sensitive uptake was calculated by subtracting the value obtained in the presence of 10 μ M 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 μ M) for 20 min and then further incubated in the presence of [³H]NE (1–30 μ M) with or without PTZ for 12 min. The apparent Michaelis constant (K_m) and the maximal velocity (V_{max}) for initial rates of [³H]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 [^3H]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 [^3H]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 [^3H]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 [^3H]nisoxetine was defined as the binding inhibited by the selective NET inhibitor nisoxetine (10 μM). ii) For [^3H]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 [^3H]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 $\times g$ 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, California, 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 $\mu\text{g}/\text{ml}$ 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 [^3H]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 $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{CaCl}_2$ 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 \pm S.E.M. Data were statistically evaluated by Student's *t*-test or one-way analysis of variance (ANOVA). If a significant *F* value was found, Dunnett's test for multiple compari-

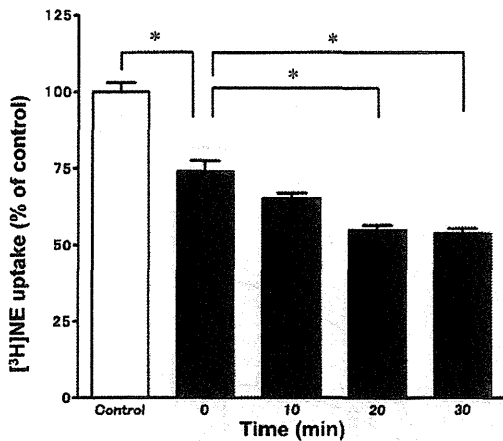


Fig. 1. Time course of effect of (-)-PTZ on [³H]NE uptake in cultured bovine adrenal medullary cells. After preincubation with or without (-)-PTZ (30 μ M) for the indicated period, the cells were incubated with or without (-)-PTZ (30 μ M) at 37°C for another 12 min in the presence of [³H]NE (500 nM). The desipramine-sensitive [³H]NE uptake by the cells was measured. Data are means \pm 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 μ M) during all procedures. The values of the control [³H]NE uptake was 0.23 ± 0.02 pmol / 10^6 cells per min. * $P < 0.05$, compared with the control or 0 min.

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

Results

(-)-PTZ (30 μ M) inhibited [³H]NE uptake by cultured bovine adrenal medullary cells in a time-dependent manner (Fig. 1). Preincubation of cells with (-)-PTZ (30 μ M) caused a decrease in [³H]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 [³H]NE uptake was performed using cells pretreated with PTZ for 20 min. Treatment with (-)- or (+)-PTZ (3 – 100 μ M) significantly inhibited [³H]NE uptake in a concentration-dependent manner (Fig. 2). The half-maximal inhibitory concentrations (IC_{50}) for inhibition of [³H]NE uptake by (-)- and (+)- PTZ were calculated as 54.59 ± 2.89 μ M and 72.38 ± 2.96 μ M, respectively. Incubation of cells with increasing concentrations of [³H]NE (1 – 30 μ M) showed that [³H]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 [³H]NE uptake with little change in the apparent Michaelis constant (K_m).

To investigate the involvement of opioid receptors and

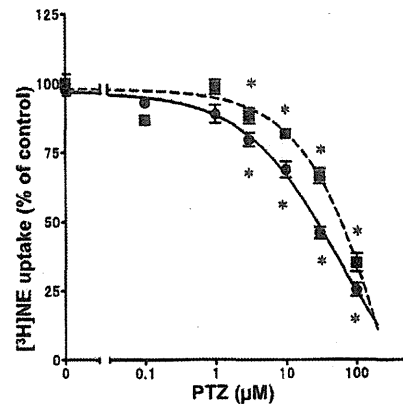


Fig. 2. Concentration-response curves of effects of (-)- and (+)-PTZ on [³H]NE uptake by cultured bovine adrenal medullary cells. After preincubation with (-)-PTZ (closed circles) or (+)-PTZ (closed squares) for 20 min, cells were incubated with 500 nM [³H]NE and various concentrations (0.1 – 100 μ M) of PTZ for 12 min at 37°C. The desipramine-sensitive [³H]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 μ M PTZ.

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 [³H]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 [³H]NE uptake reduced by PTZ. Inhibitors of protein kinase C (calphostin C, chelerythrine, and GF109203X) did not affect the PTZ-induced inhibition of [³H]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 [³H]nisoxetine, a specific inhibitor of NET, to plasma membranes isolated from bovine adrenal medulla. The specific binding of [³H]nisoxetine to plasma membranes was saturable with an increasing concentration of 2 – 32 nM [³H]nisoxetine, although (-)- and (+)-PTZ (30 μ M) did not inhibit [³H]nisoxetine binding (Fig. 6). Scatchard plot analysis showed that (-)- and (+)-PTZ (30 μ M) 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 [³H]nisoxetine specific binding to intact bovine adrenal

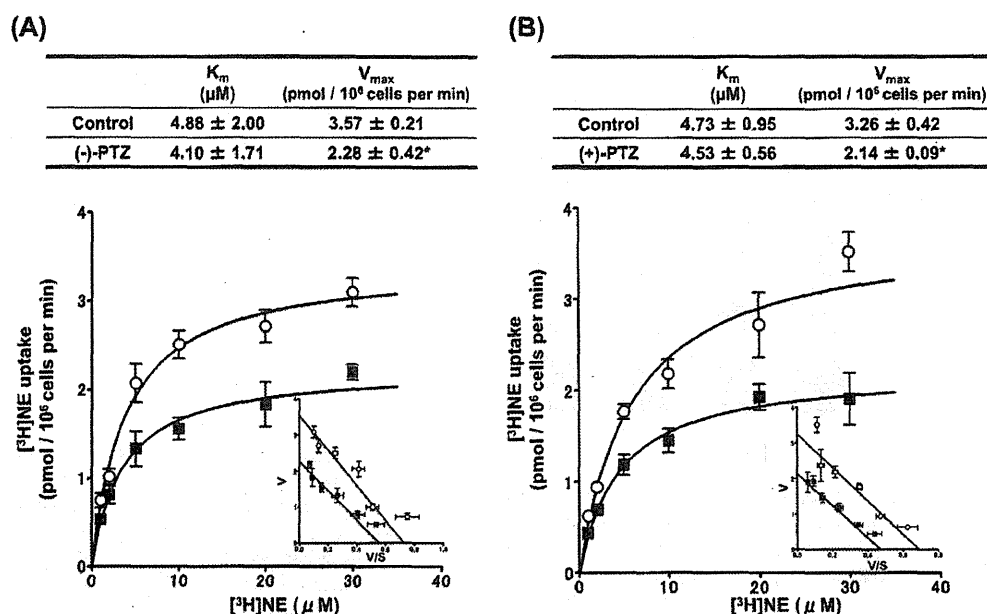


Fig. 3. Saturation curve of [^3H]NE uptake and its Eadie-Hofstee analysis. After preincubation with (closed squares) or without (open circles) (-)-PTZ (A) and (+)-PTZ (B) ($30 \mu\text{M}$) for 20 min, cells were incubated with various concentrations ($1 - 30 \mu\text{M}$) of [^3H]NE in the presence (closed squares) or absence (open circles) of (-)-PTZ (A) and (+)-PTZ (B) ($30 \mu\text{M}$) for 12 min at 37°C . [^3H]NE uptake was measured. Lower inset: Eadie-Hofstee analysis of [^3H]NE uptake. Data are means \pm S.E.M. of three separate experiments carried out in duplicate. V, velocity ($\text{pmol} / 10^6 \text{ cells per min}$); V/S, velocity / substrate concentration ($\text{pmol} / 10^6 \text{ cells} \cdot \text{min} \cdot \mu\text{M}$). * $P < 0.05$, 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 [^3H]nisoxetine ($2 - 64 \text{ nM}$) to SK-N-SH cells and reduced the B_{max} of [^3H]nisoxetine binding without any change in the K_d (Fig. 7). (-)-PTZ ($1.0 - 100 \mu\text{M}$) suppressed the specific binding of [^3H]nisoxetine to SK-N-SH cells in a concentration-dependent manner (Fig. 8A) similar to that of [^3H]NE uptake by the cells (Fig. 8B). To examine whether (-)-PTZ-induced inhibition of [^3H]NE uptake or [^3H]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 [^3H]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 $^{45}\text{Ca}^{2+}$ influx after treatment of cells with PTZ. (-) and (+)-PTZ ($30 \mu\text{M}$), however, had little effect on 56 mM K^+ -induced $^{45}\text{Ca}^{2+}$ 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 [^3H]NE uptake in a concentration ($3.0 - 100 \mu\text{M}$)-dependent manner. The inhibitory effect of (+)-PTZ ($\text{IC}_{50} = 72.4 \mu\text{M}$) was slightly less potent than that of (-)-PTZ