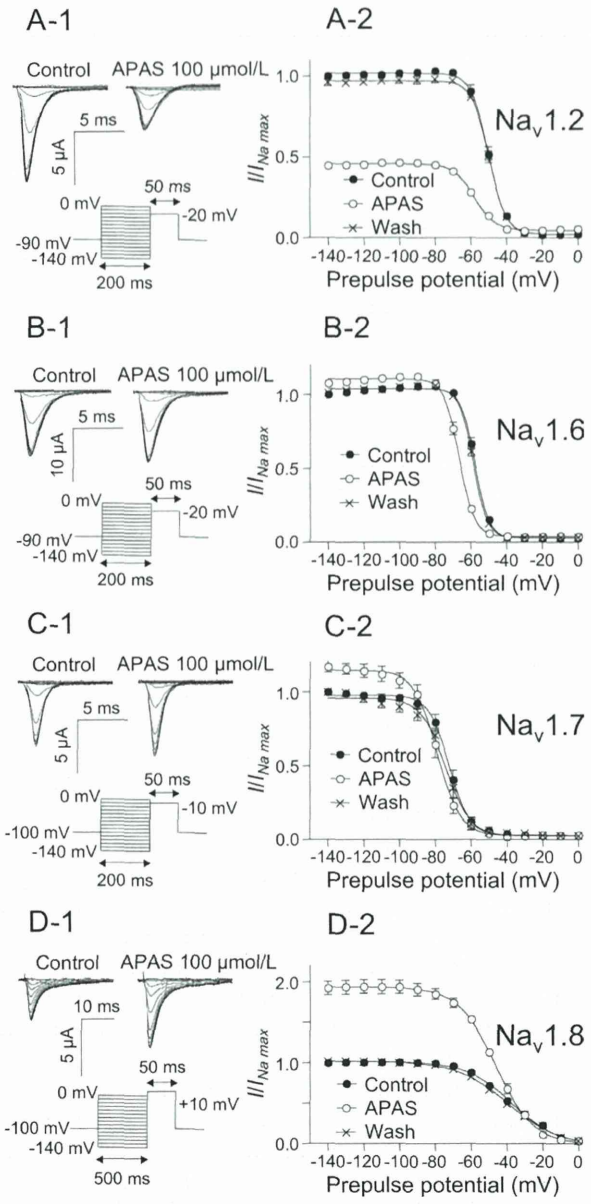

Fig. 7. Effects of allopregnanolone sulfate (APAS) on inactivation curves in oocytes expressing $Na_v1.2$ (*A*) (n = 6), $Na_v1.6$ (*B*) (n = 7), $Na_v1.7$ (*C*) (n = 5), or $Na_v1.8$ (*D*) (n = 6) α subunits with β_1 subunits. Currents were elicited by a 50-ms test pulse to −20 mV for $Na_v1.2$ and $Na_v1.6$, −10 mV for $Na_v1.7$, and +10 mV for $Na_v1.8$ after 200 ms (500 ms for only $Na_v1.8$) prepulses ranging from −140 mV to 0 mV in 10-mV increments from a V_{max} holding potential. Representative I_{Na} traces in both the absence and presence of APAS are shown in *A-1*, *B-1*, *C-1*, and *D-1*. Effects of APAS on inactivation curves (*closed circles*, control; open circles, neurosteroids; cross, washout) are shown in *A-2*, *B-2*, *C-2*, and *D-2*. Steady-state inactivation curves were fitted to the Boltzmann equation, and the $V_{1/2}$ values are shown in table 2. Data are expressed as means ± SEM. Na_v = voltage-gated sodium channel; Wash = washout.

sodium currents in the hyperpolarizing range of the inactivation curve, indicating that resting channel block is an important mechanism of APAS inhibition for only $Na_v1.2$. Both compounds demonstrated use-dependency for inhibition of $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$, suggesting the ability to slow the recovery time from inactivation.[33] Many investigators have shown that sodium channel blockers, including local anesthetics, tricyclic antidepressants, and volatile anesthetics, enhance steady-state inactivation with no effect on activation and exhibit use-dependent block.[34–36] We demonstrated that APAS enhances inactivation and shows use-dependent block similar to other sodium channel blockers, yet it also has diverse effects on activation according to differences in α subunits. These actions suggest that APAS may have different binding sites or allosteric conformational mechanisms to change sodium channel function, although further investigation with site-directed mutagenesis is needed to rule out nonspecific membrane effects. PAS may have common binding sites with APAS, because it shows similar effects, although these changes were small.

The α subunit consists of four homologous domains (I to IV) containing six transmembrane segments (S1 to S6), and one reentrant P-region connecting S5 to S6 (SS1/SS2). Tetrodotoxin-sensitive α subunits, $Na_v1.2$, $Na_v1.6$, and $Na_v1.7$, are phylogenetically related and show 70 to 80% amino acid sequence identity. In contrast, tetrodotoxin-resistant α subunits, $Na_v1.8$, are phylogenetically distant and show only 55 to 56% sequence identity to the other three α subunits. In addition, the lengths of amino acid sequences of four α subunits differed within the range of 1957 to 2005 residues. Therefore, these differences would result in the diversity in neurosteroid action, especially in the effects on channel activation. Indeed, the longest extracellular regions in the α subunit (IS5 to SS1) are 93, 77, 73, and 66 amino acid residues in $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$, respectively. The diversity in sequence and differences in the effects on activation according to α subunit may be important for clarifying binding sites and the mechanism of $Na_v1.2$ inhibition by APAS in further investigations.

γ-Aminobutyric acid type A receptors have been considered to be important for the analgesic effects of allopregnanolone because it has high potency as a positive $GABA_A$ modulator compared with other neurosteroids. Pregnanolone also affects $GABA_A$ receptors in a manner similar to that of allopregnanolone; nevertheless, its analgesic effect is weak. In fact, pregnanolone was shown to reduce mechanical allodynia without reduction of thermal heat hyperalgesia in a neuropathic pain model in contrast to attenuation of both by allopregnanolone.[28] The investigators suggested that the partial analgesic effects of pregnanolone are caused by suppression of glycine receptors by demonstrating that pregnanolone had a significant analgesic effect only in animals displaying a strychnine-induced allodynia in two types of allodynia models induced by bicuculline and strychnine.[28] Moreover, a recent report demonstrated that

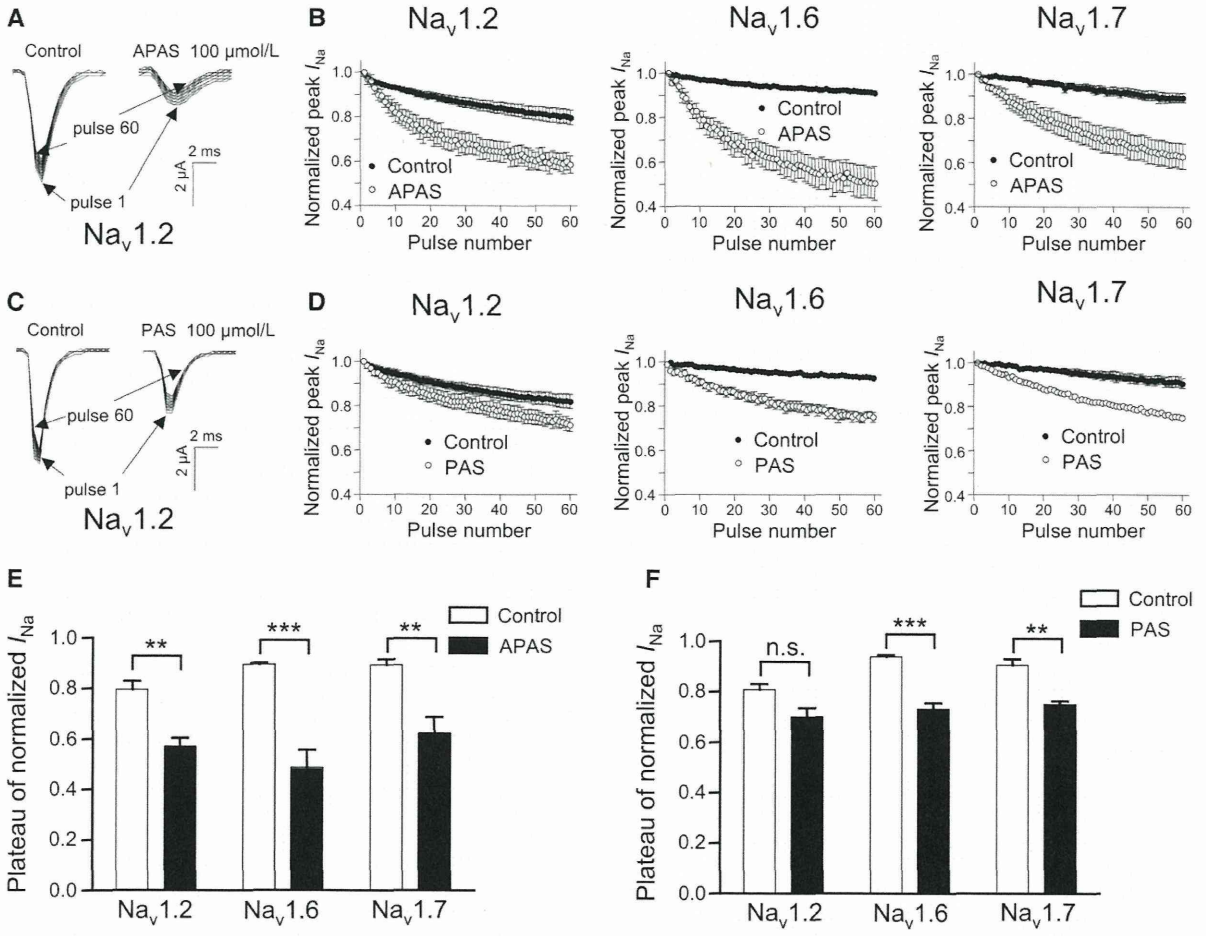

Fig. 8. Use-dependent blockage of sodium channels on Na$_v$1.2 (n = 5), Na$_v$1.6 (n = 6), and Na$_v$1.7 (n = 5) α subunits with β_1 subunits by allopregnanolone sulfate (APAS) and pregnanolone sulfate (PAS). Currents were elicited at 10 Hz by a 20-ms depolarizing pulse of −20 mV for Na$_v$1.2 and Na$_v$1.6 and −10 mV for Na$_v$1.7 from a $V_{1/2}$ holding potential in both the absence and presence of 100 μmol/l of the two compounds; representative I_{Na} traces in both the absence and presence of the two compounds (*A* and *C*). Peak currents were measured and normalized to the first pulse and plotted against the pulse number (*B*, the effects of APAS; *D*, the effects of PAS). *Closed circles* and *open circles* represent control and the effect of neurosteroids, respectively. Data were fitted to the monoexponential equation, and values for fractional blockage of the plateau of normalized I_{Na} are shown in *E* and *F*. Data are expressed as means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with the control, based on paired *t* test (two-tailed). Na$_v$ = voltage-gated sodium channel.

allopregnanolone shows analgesic effects in rats through suppression of T-type Ca^{2+} currents and potentiation of $GABA_A$ currents.[16] These previous reports indicate several mechanisms underlying the analgesic effect of allopregnanolone likely exist, as well as potentiation of $GABA_A$ receptors.

Sodium channel α subunits expressed in the dorsal root ganglion (Na$_v$1.7, Na$_v$1.8, and Na$_v$1.9) are thought to be involved in the pathogenesis of inflammatory and neuropathic pain. A recent study reported that Na$_v$1.2 also plays an important role in pain signaling. It was reported that Na$_v$1.2 and Na$_v$1.3 predominantly compose functional sodium channel currents within lamina I/II (dorsal horn) neurons, which mediate acute and chronic nociceptive signals from peripheral nociceptors to pain-processing regions in the brain.[37] Another recent report showed that mutations in Na$_v$1.2 are associated with seizures and pain characterized by headaches and back pain.[38] A disubstituted succinamide, a potent sodium channel blocker, was reported to attenuate nociceptive behavior in a rat model of tonic pain and was demonstrated to potently block Na$_v$1.2, as well as Na$_v$1.7 and Na$_v$1.8, with a potency two orders of magnitude higher than anticonvulsant and antiarrhythmic sodium channel blockers currently used to treat neuropathic pain.[39] Other investigators demonstrated that four sodium channel blockers, including lidocaine, mexiletine, benzocaine, and ambroxol, which are used clinically to treat pain, suppressed recombinant Na$_v$1.2 currents as well as tetrodotoxin-resistant Na^+ channel currents in rat sensory neurons, which comprised mostly Na$_v$1.8 currents. The authors suggested that these sodium channel blockers would induce analgesia according

to the amount of sodium channel blocking, including $Na_v1.2$ and $Na_v1.8$.[40] These recent reports support that suppression of $Na_v1.2$ function by APAS might be a mechanism underlying the analgesic effects of allopregnanolone.

In conclusion, APAS and PAS have diverse effects on $Na_v1.2$, $Na_v1.6$, $Na_v1.7$, and $Na_v1.8$ α subunits expressed in *Xenopus* oocytes, with differences in the effects on sodium channel gating. In particular, only APAS inhibited sodium currents of $Na_v1.2$ at pharmacologically relevant concentrations. These results raise the possibility that suppression of $Na_v1.2$ by APAS may be important for pain relief by allopregnanolone and provide a better understanding of the mechanisms underlying the analgesic effects of allopregnanolone. However, further studies are needed to clarify the relevance of sodium channel inhibition by APAS.

Acknowledgments

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Horishita: Department of Anesthesiology, School of Medicine, University of Occupational and Environmental Health, 1-1 Isegaoka, Yahatanisiku, Kitakyushu 807–8555, Japan. thori@med.uoeh-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

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V. 化学物質リスク研究事業・班会議資料

平成 26 年 11 月 1 日開催

平成 26 年 12 月 3 日開催

平成 27 年 1 月 31 日開催

平成26年度厚生労働科学研究費補助金

化学物質リスク研究事業

「個体の成長期における毒性メカニズムに基づく新規in vitro発達神経毒性評価法に関する研究」

班会議　議事録

日時:平成 26 年 11 月 1 日(土)11 時 00 分～18 時 15 分

場所:国立医薬品食品衛生研究所　4 号館 2 階　薬理部部長室

(〒158-8501　東京都世田谷区上用賀1-18-1)

出席者:上野　晋、笛田由紀子(産業医大)、吉田祥子(豊橋技術科学大)、関野祐子、諫田泰成(国衛研)　(以上、敬称略、順不同)

議事:

1. はじめに　(11:00～11:15)

本年度の研究班の進め方について(諫田)

2. in vitro 評価系の進捗状況(11:15～12:30)

諫田「ヒト未分化細胞を用いた発達期毒性評価系の構築」

(ランチ・休憩)

3. 2 年目の研究班共通の陽性対照物質の選定(13:15～15:30)

4. in vivo 評価系の進捗状況　(15:30～18:15)

① 関野・吉田

「生後神経回路の機能的影響評価指標に関する研究」

② 上野・笛田

「幼若期の神経回路機能に対する化学物質の影響評価－BMI への感受性－」

以上

平成26年11月1日
化学物質リスク事業・班会議

ヒト未分化細胞を用いた発達期毒性評価系の構築

国立医薬品食品衛生研究所
薬理部第二室
諫田　泰成

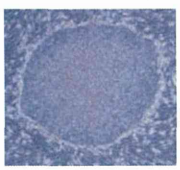
内容

1. ヒアリング資料の再確認
2. 有機スズのin vitro作用

ヒト未分化細胞のモデル

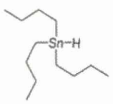
- ヒト胎児性癌細胞株NT2/D1

- ヒトiPS細胞



陽性対照物質：トリブチルスズ（TBT）

- ✓ 内分泌攪乱作用をもつ環境汚染物質。
- ✓ 低濃度の曝露により、神経系や免疫系など様々な細胞毒性を引き起こす。
- ✓ TBTを投与された妊娠ラットから生まれたF1は、行動異常を示す。
- ✓ ヒト発達期に対する影響はいまだ明らかではない。

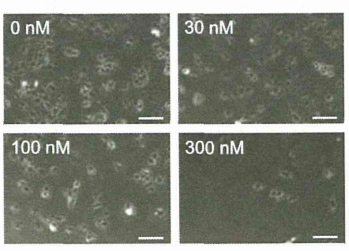


ヒト未分化細胞の「エネルギー代謝」に着目し、
TBT曝露による毒性メカニズムを解析した。

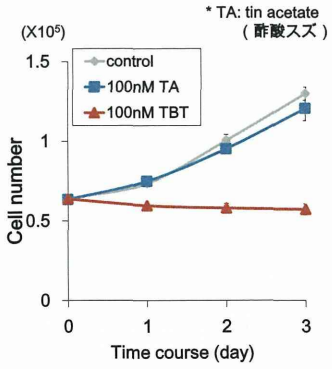
低濃度TBT曝露による増殖抑制

ヒト胎児性癌細胞 (NT2)

TBT曝露 (血清存在下、24時間) による細胞数の変化



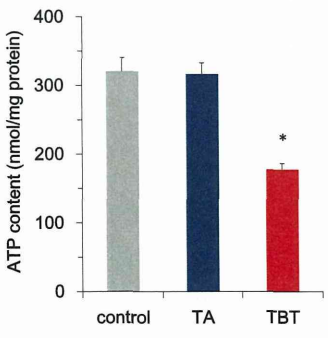
(Bar = 100 μm)



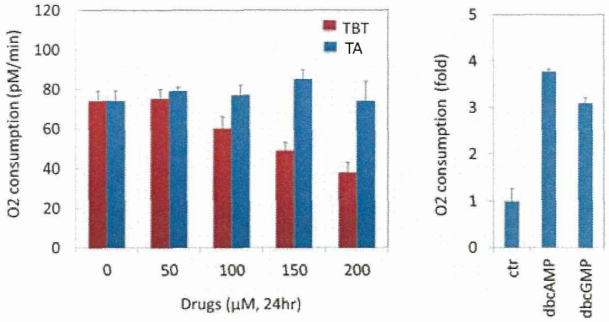
Yamada et al, Metallomics, 2013

低濃度TBTによる細胞内ATP量の低下

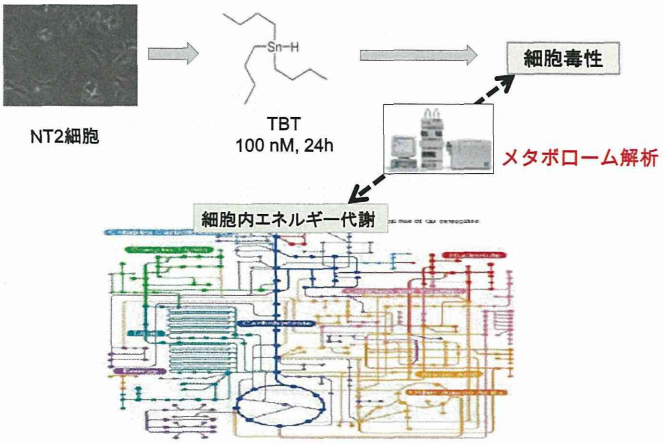
100nM TBTで24時間曝露
↓
0.1% Triton-Xにて細胞を溶解
↓
ルシフェリンとルシフェラーゼを添加
↓
ルミノメーターによる発光強度の測定



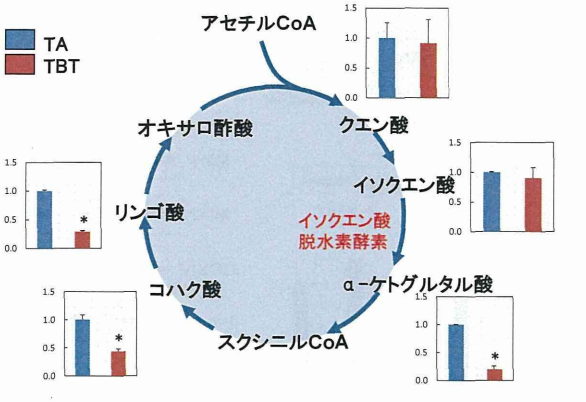
TBT曝露による酸素消費量の抑制



メタボローム法を利用したTBT毒性解析



TBTの新規作用点IDH3

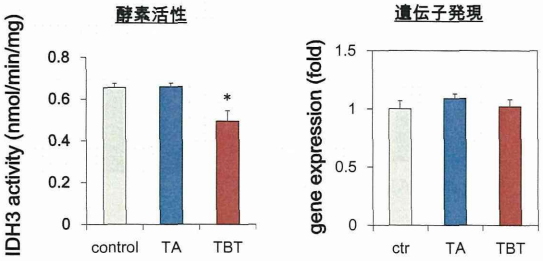


Yamada et al, Scientific Reports, 2014

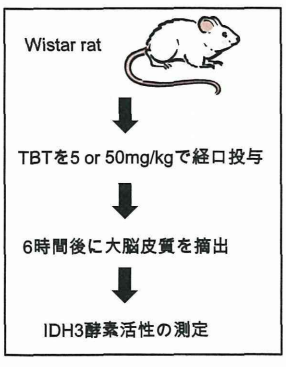
IDH3活性に対するTBT曝露の影響

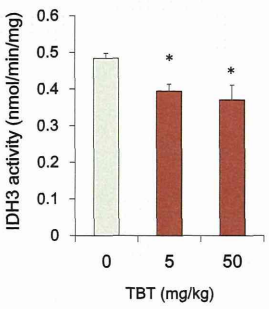
イソクエン酸脱水素酵素

	細胞内局在	補酵素	反応
IDH1	細胞質	$NADP^+$	可逆
IDH2	ミトコンドリア	$NADP^+$	可逆
IDH3	ミトコンドリア	NAD^+	不可逆

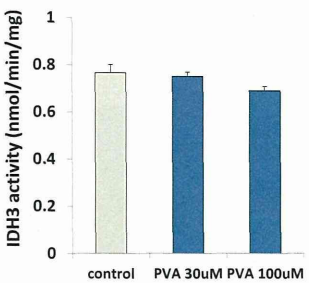


in vivoにおけるTBTの作用





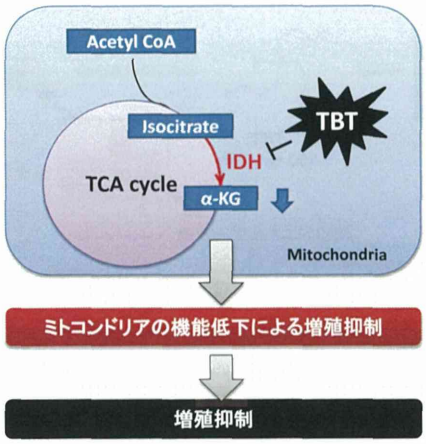
他の化学物質の影響は？



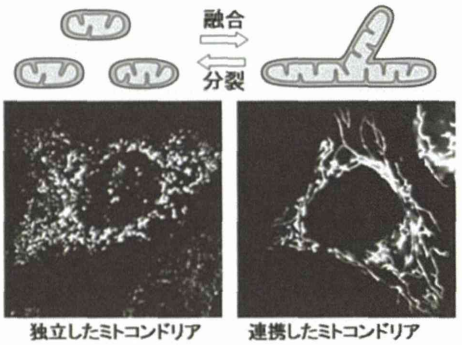
バルプロ酸処理によってもIDH3の活性が抑制される傾向であった。

IDH3を含めたエネルギー産生は高感度で毒性を検出できる可能性が考えられる。

小括

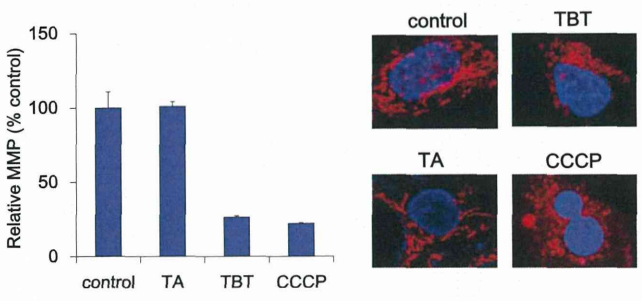


ミトコンドリアの動的制御



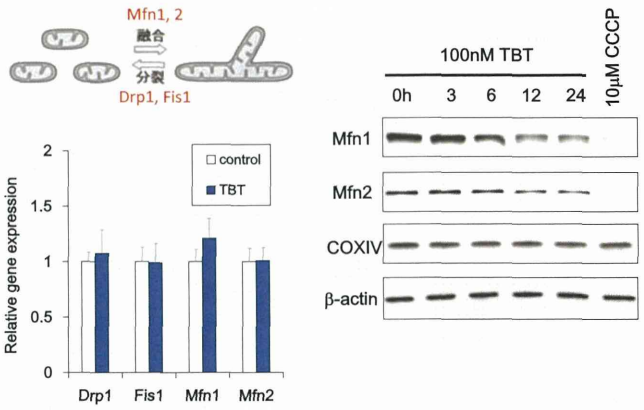
石原、生化学 83: 365-373 (2011)

低濃度TBTによるミトコンドリアの膜電位低下と形態変化



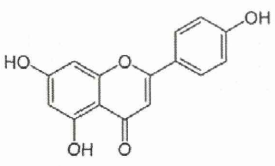
Yamada et al., in preparation

TBTによるミトコンドリア融合タンパク質の分解



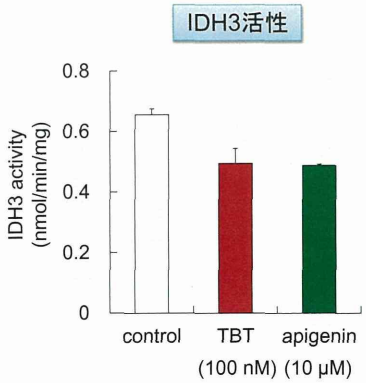
IDH3阻害剤 apigenin

apigenin

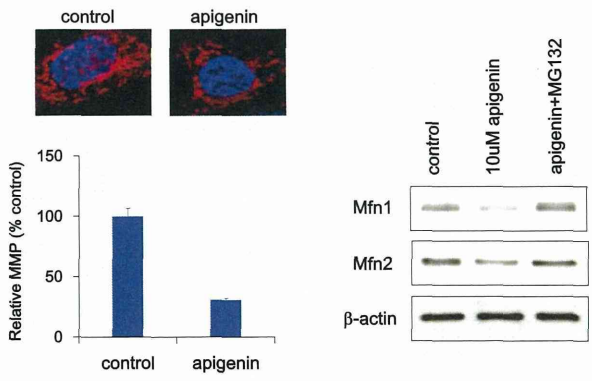


- 植物に含まれるフラボノイド
- IDH3活性を阻害することが報告されている

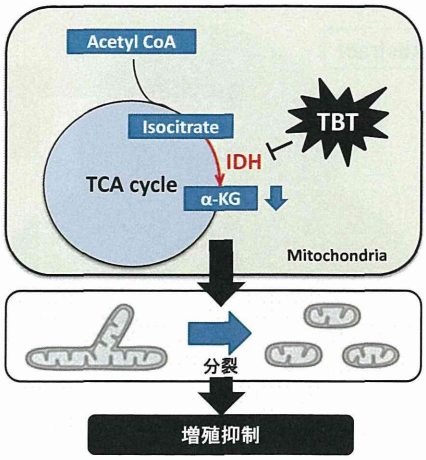
PNAS110: E2153–62 (2013)



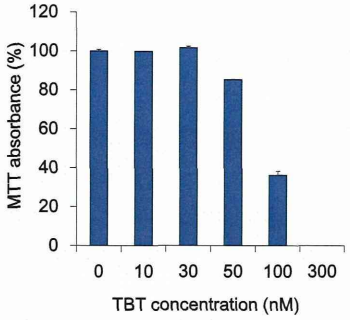
IDH3阻害剤によるミトコンドリアの膜電位低下と融合タンパク質の分解



結論



TBTによるiPS細胞の増殖抑制



まとめ

低濃度TBTは、ミトコンドリアの融合タンパク質の分解を促進してミトコンドリアの機能低下を引き起こす新たな毒性発現メカニズムが示唆された。

幹細胞におけるMfn分解にともなうATP産生は、毒性評価の有用な指標になる可能性が考えられる。