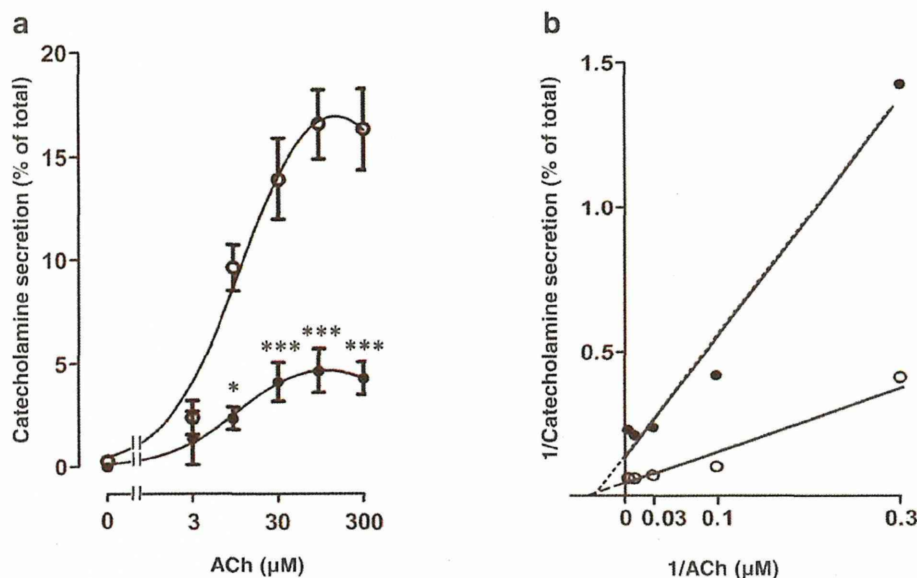

Fig. 4 Inhibitory mode of ikarisoside A on catecholamine secretion induced by ACh. **a** After preincubation for 10 min, cells were stimulated with (*black circle*) or without (*white circle*) ikarisoside A (10 μM) in the presence or absence of ACh (1–300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate. The data of ACh plus ikarisoside A are shown by subtracting basal secretion obtained in the presence of ikarisoside A. $*P < 0.05$ and $***P < 0.001$, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test). **b** Double-reciprocal plot analysis of the data in (**a**)

ikarisoside A seems to inhibit nAChR-ion channels but not voltage-dependent Na^+ channels or voltage-dependent Ca^{2+} channels. Ikarisoside A inhibited Ca^{2+} influx and Na^+ influx induced by ACh in a concentration-dependent manner similar

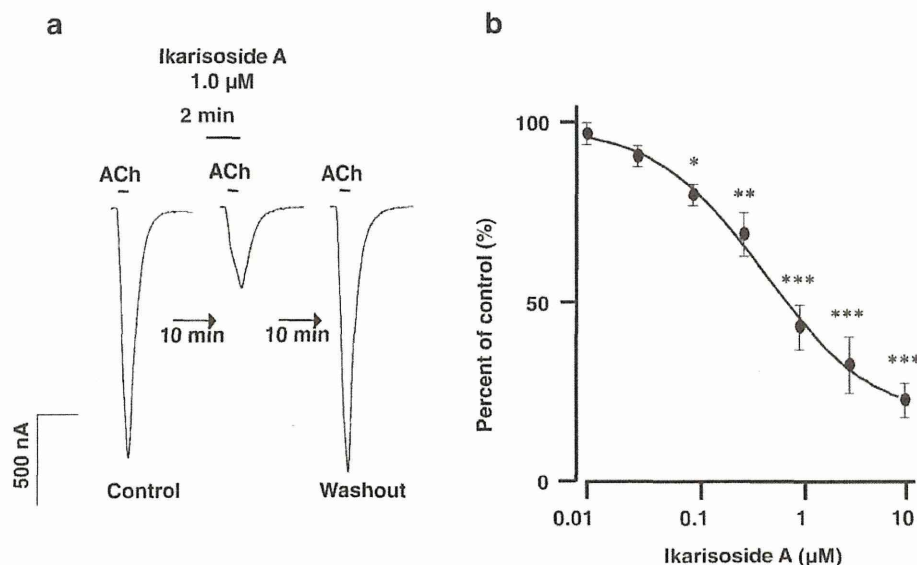

Fig. 5 Effects of ikarisoside A on peak ACh-induced inward currents in *Xenopus* oocytes expressing rat α3β4 nAChRs. **a** Representative traces from a single *Xenopus* oocyte are shown. The currents of ikarisoside A-treated oocytes were recorded 10 min after recording of the control currents, and the washout currents were obtained 10 min after ikarisoside A treatment. Ikarisoside A (1 μM) suppressed the currents induced by the EC_{50} (0.2 mM) of ACh, and the inhibitory effects were reversible. **b** Concentration-response curve for the inhibitory effects of ikarisoside A on ACh-induced currents. The peak current amplitude in the presence of ikarisoside A was normalized to that of the control and the effects are expressed as percentages of the control. Data are presented as means ± SEM from four separate experiments carried out in triplicate. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, compared to the control (based on one-way ANOVA with Dunnett's multiple comparison post hoc test). Nonlinear regression analysis was performed and the mean value of IC_{50} for ikarisoside A is 0.48 μM

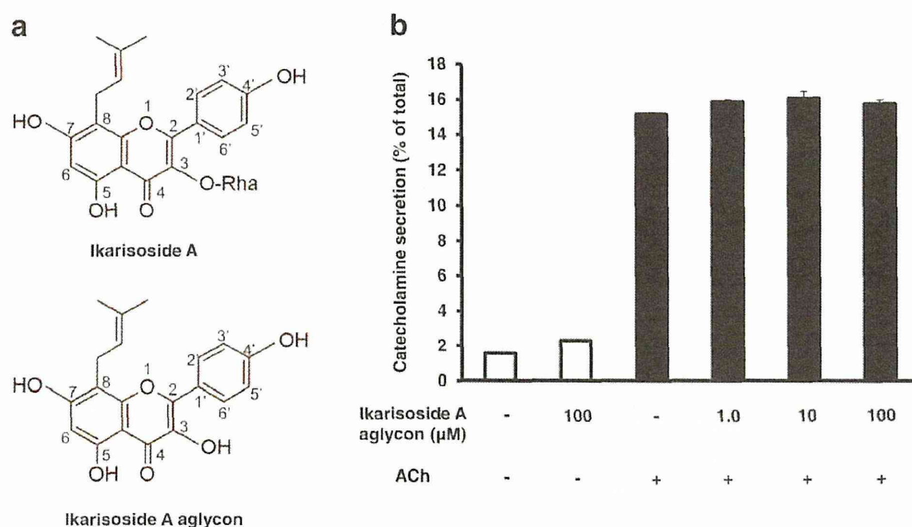

Fig. 6 Structure of ikarisoside A and its aglycon (**a**) and effect of aglycon of ikarisoside A on ACh-induced catecholamine secretion (**b**). **a** Structure of ikarisoside A and its aglycon (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-enyl)-4*H*-chromen-4-one). **b** After preincubation with cells with or without aglycon of ikarisoside A (1–100 μM) for 10 min, the cells (10^6/well) were incubated with or without aglycon of ikarisoside A (1–100 μM) and ACh (300 μM) for another 10 min at 37 °C. Catecholamines secreted into the medium were expressed as a percentage of the total catecholamines in the cells. Data are means ± SEM from three separate experiments carried out in triplicate

to that of catecholamine secretion. In the exocytotic secretion of catecholamines, Ca^{2+} plays an indispensable role as the coupler in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). From these findings, it is likely that ikarisoside A inhibits ACh-induced catecholamine secretion by suppressing nAChR-ion channels. We investigated the inhibitory mode of ikarisoside A on nAChR-ion channels. Even when the concentration of ACh was increased, the inhibitory

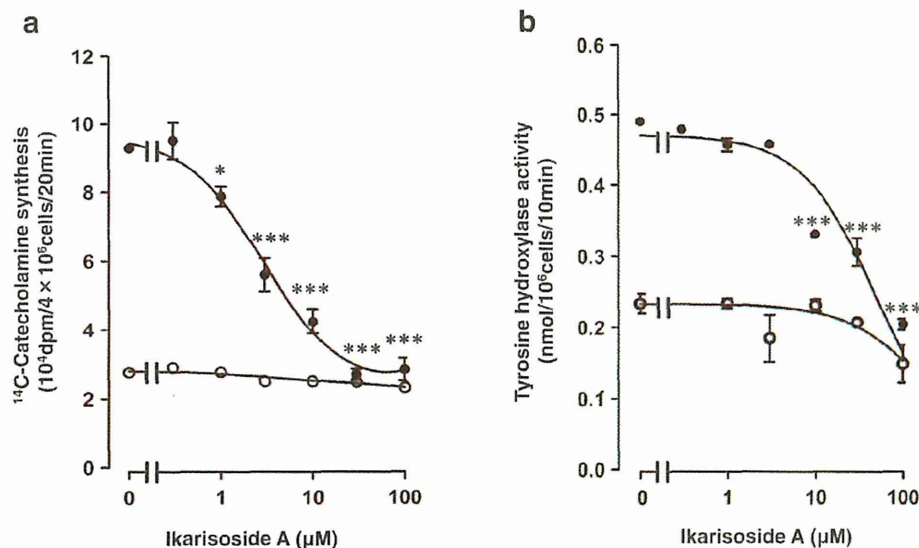

Fig. 7 Effects of ikarisoside A on ^{14}C-catecholamine synthesis from [^{14}C]tyrosine (**a**) and tyrosine hydroxylase activity (**b**) in the cells. **a** After preincubation for 10 min with or without ikarisoside A (0.1–100 μM), cells (4 × 10^6/dish) were incubated with L-[U-^{14}C] tyrosine (20 μM, 1 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 20 min. The ^{14}C-catecholamines formed were measured. **b** After preincubation with or without ikarisoside A (0.1–100 μM) for 10 min, cells (10^6/well) were incubated with L-[1-^{14}C] tyrosine (18 μM, 0.2 μCi) in the presence or absence of ikarisoside A (0.1–100 μM) and with (*black circle*) or without (*white circle*) 300 μM ACh at 37 °C for 10 min, and tyrosine hydroxylase activity was measured. Data are means ± SEM from three separate experiments carried out in triplicate. *P < 0.05 and ***P < 0.001, compared with ACh alone (analyzed by one-way ANOVA with Dunnett's multiple comparison post hoc test)

effect of ikarisoside A on ACh-induced secretion of catecholamines was not overcome, suggesting a noncompetitive inhibition and that ikarisoside A acts at a site different from that for ACh binding. A previous review (Lena and Changeux 1993) reported that the site at which noncompetitive blockers act lies at the interface between the nicotinic receptor protein and the membrane lipids.

In the *Xenopus* oocytes expressed with α3β4 nAChRs, ikarisoside A directly inhibited ACh-induced current. The IC_{50} values of ikarisoside A for $^{22}Na^+$ influx in adrenal medullary cells and for Na^+ current in the oocytes were 2.96 and 0.48 μM, respectively. The IC_{50} in the bovine adrenal medullary cells is 6.2-fold bigger than that of the drug in the oocyte system. Although the reason for the discrepancy of the IC_{50} between the two systems is not yet clear, the discrepancy may be explained in the following way. (1) A maximally effective concentration of ACh was used for the $^{22}Na^+$ influx experiments in bovine adrenal medullary cells but the half-maximal concentration was used for the Na^+ current in the oocyte system. (2) In the oocyte expression system, there may be some changes in the test compound potency compared to that of the method using mammalian cells, i.e., a decrease (Lambert et al. 2001; Akk et al. 2008) or an increase (Pintado et al. 2000) in the sensitivity of test compounds. (3) Bovine adrenal medullary cells express multiple nAChR subtypes such as α3β4 (Criado et al. 1992; Garcia-Guzman et al. 1995), α3β4α5 (Campos-Caro et al. 1997), and α7 (Lopez et al. 1998). We should study above possibilities and examine the effect of ikarisoside A on the function of nAChRs in other mammalian cells.

Structure-activity relationship of ikarisoside A for inhibition of nAChR-ion channels

In the present study, we used four flavonol glycosides derived from the *Epimedium* species. Ikarisoside A, but not the other three flavonols, inhibited the functioning of nAChR-ion channels. Judging from the differences in their structures, ikarisoside A has a hydroxyl group at the 7 position in the structure whereas other three have a glucose moiety at this position, suggesting that a glucose moiety at the 7 position may induce stereo-specific interference when flavonol glycosides interact with nAChRs. Furthermore, the inhibition of ACh-induced secretion by ikarisoside A disappeared by the removal of the rhamnose moiety at the 3 position from ikarisoside A. These findings suggest that the rhamnose moiety at the 3 position of ikarisoside A is essential to inhibit the function of nAChR-ion channels.

Inhibitory effect of ikarisoside A on catecholamine synthesis

Ikarisoside A inhibited not only catecholamine secretion but also reduced catecholamine synthesis in ACh-stimulated cells. In the regulation of catecholamine synthesis, Ca^{2+} plays an important role as the coupler in the stimulus-synthesis coupling (Yanagihara et al. 1987) as well as in the stimulus-secretion coupling (Douglas and Rubin 1961, 1963). In the present study, we observed that ikarisoside A suppressed the $^{22}Na^+$ influx and the subsequent $^{45}Ca^{2+}$ influx by inhibiting nAChR-ion channels. Therefore, it is likely that ikarisoside A inhibits catecholamine synthesis and tyrosine hydroxylase activity induced by ACh via the suppression of Ca^{2+} influx in cultured bovine adrenal medulla cells. In harmony with this view, the IC_{50} values of ikarisoside A for inhibition of $^{22}Na^+$ and $^{45}Ca^{2+}$ influx and for inhibition of catecholamine synthesis and tyrosine hydroxylase are very similar.

Pharmacological significance of the inhibitory effects of ikarisoside A on adrenal medullary functions

The human serum concentration of ikarisoside A has not been reported yet. Several previous in vitro studies reported that ikarisoside A at 5.0–20 μM inhibits osteoclastogenic differentiation and nitric oxide synthase in murine monocyte/macrophage cell line RAW264.7 cells (Choi et al. 2008, 2010) and induces neurite outgrowth activity in PC12h cells at 10 μM (Kuroda et al. 2000). In the present study, we observed a significant inhibition of ikarisoside A at 0.1 and 1.0 μM in ACh-induced current in *Xenopus* oocytes and ACh-induced synthesis and secretion of catecholamines, respectively.

It is well known that adrenaline and noradrenaline have an important role in the regulation of normal function in the central and peripheral sympathetic nervous systems. Under strong and prolonged stress, an increased catecholamine release may occur, which possibly induces cardiovascular diseases such as hypertension, atherosclerosis, coronary heart disease, and heart failure (Yanagihara et al. 2014). Chronic heart failure is reported to be associated with the activation of the sympathetic nervous system as manifested by increased circulating catecholamines (Westfall and Westfall 2011). Furthermore, Hara et al. (2011) reported that the stress hormone adrenaline stimulates β_2-adrenoceptors, which activates the Gs protein/cyclic AMP-dependent protein kinase and the β-arrestin-mediated signaling pathway, reduces the p53 level, and induces DNA damage.

Our previous studies reported that daidzein, a soy isoflavone, (Liu et al. 2007) and nobiletin, a citrus polymethoxy flavone, (Zhang et al. 2010) suppress the secretion and synthesis of catecholamines induced by ACh in cultured bovine adrenal medullary cells. In addition to these flavonoids, ikarisoside A also may protect the hyperactive catecholamine system induced by strong stress or emotional excitation which evokes the secretion of ACh from the splanchnic nerves. Further in vivo experiments will provide more conclusive information on ikarisoside A and promote the development of a

therapeutic drug for stress-induced disorders associated with mental or cardiovascular diseases.

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Conflict of interest The authors declare that they have no competing interests.

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V. 化学物質リスク研究事業・班会議資料

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平成 26 年 2 月 6～7 日開催

(in vivo 研究グループ)

平成 26 年 11 月 1 日開催

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化学物質リスク研究事業・班会議資料

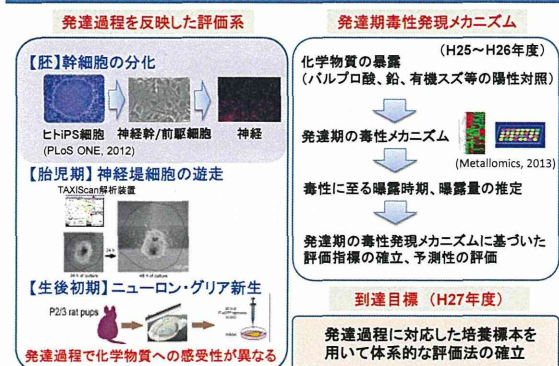
H25 年度

平成25年8月23日
化学物質リスク事業・班会議（八重洲倶楽部）

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

胎生・神経発達期の評価系



神経毒性評価の流れ

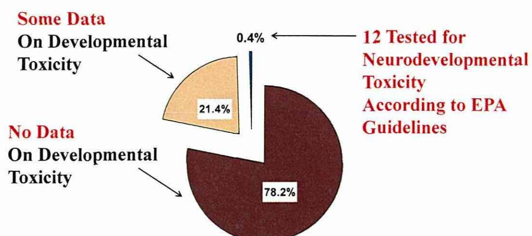
Most Chemicals Have Not Been Adequately Tested for Toxicity

- 80,000+ chemicals in commerce
- 2,863 produced or imported in quantities of 1 million pounds or more per year (High Production Volume; HPV)
- No basic toxicity information is publicly available for about half of HPV chemicals
- Information on developmental toxicity is publicly available for about 20% of HPV chemicals

EPA: Chemical Hazard Data Availability Study, 1998

Status of Developmental Toxicity Testing for the 2,863 Chemicals

Produced *Above 1 million pounds/year*



現在の発達毒性試験法の問題点

1. 時間・コストがかかりすぎる
2. 大規模なスクリーニングに向かない
3. ヒトと動物の「種差」の問題

現在の発達毒性試験法の問題点

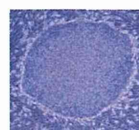
1. 時間・コストがかかりすぎる
2. 大規模なスクリーニングに向かない
3. ヒトと動物の「種差」の問題

ヒトのモデル細胞を用いたin vitroアッセイ系により
発達期の神経毒性を評価できないだろうか？

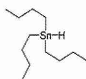
ヒト未分化細胞のモデル

- ヒト胎児性癌細胞株NT2/D1

- ヒトiPS細胞由来の神経幹細胞



陽性対照物質：トリブチルスズ（TBT）



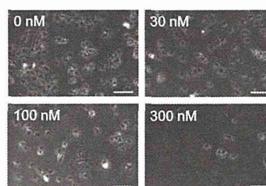
- ✓ 内分泌攪乱作用をもつ環境汚染物質。
- ✓ 低濃度の曝露により、神経系や免疫系など様々な細胞毒性を引き起こす。
- ✓ TBTを投与された妊娠ラットから生まれたF1は、行動異常を示す。
- ✓ ヒト発達期に対する影響はいまだ明らかではない。

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。

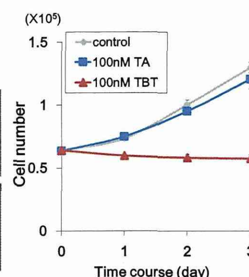
低濃度TBT曝露による増殖抑制

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

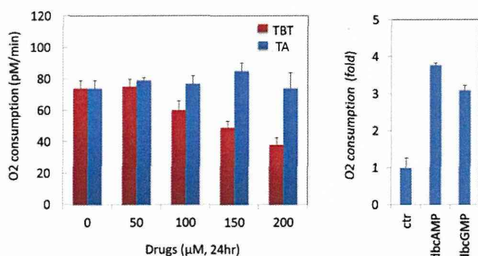


(Bar = 100 μm)

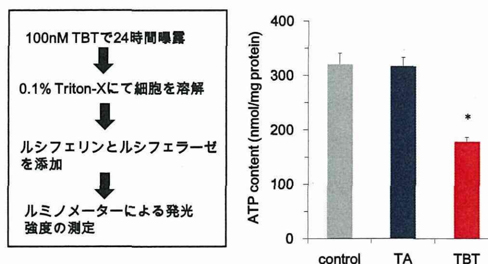


* TA: tin acetate（酢酸スズ）

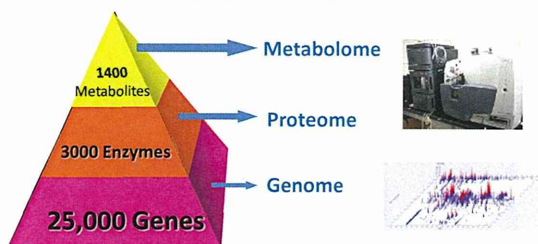
TBT曝露による酸素消費量の抑制



TBT曝露による細胞内ATP量の低下

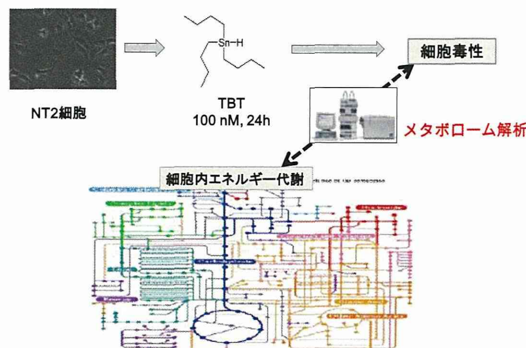


メタボローム解析

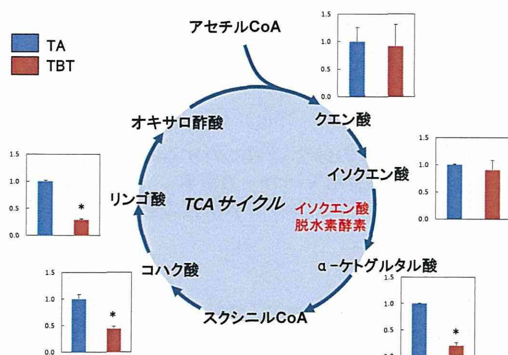


細胞全体の働きを理解するためには、ゲノム、プロテオームに加えて、アミノ酸など代謝物質の網羅的解析が有用。

メタボローム法を利用したTBT毒性解析



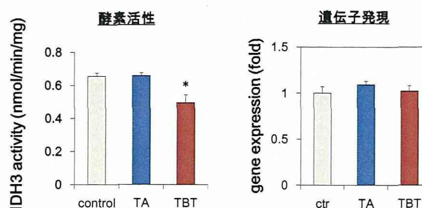
TCAサイクルに対するTBT曝露の影響



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



IDH2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
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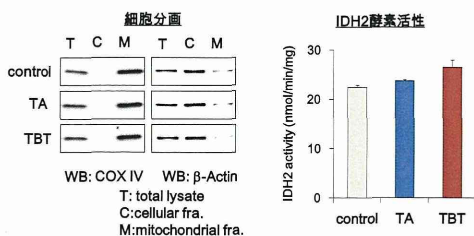
IDH3	ミトコンドリア	NAD+	不可逆



細胞増殖に対するIDH3αノックダウンの影響