Mechanism of liver-selective thyromimetic activity of SK&F L-94901: evidence for the presence of a cell-type-specific nuclear iodothyronine transport process

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Abstract

The thyromimetic compound SK&F L-94901 shows more potent thyromimetic activity in the liver than in the pituitary gland or heart when administered to rats. The mechanisms of liver-selectivity of SK&F L-94901 were examined using cultured rat hepatoma cells (dRLH-84) and rat pituitary tumor cells (GH3), both of which showed saturable cellular uptake of tri-iodothyronine (T_3). When isolated nuclei with partial disruption of the outer nuclear membrane were used, SK&F L-94901 competed for [125 I] T_3 binding to nuclear receptors almost equally in dRLH-84 and GH3 cells. SK&F L-94901 also did not discriminate thyroid hormone receptors (TR) $\alpha 1$ and $\beta 1$ in terms of binding affinity and activation of the thyroid

hormone responsive element. In intact cells, however, SK&F L-94901 was a more potent inhibitor of nuclear [125 I] T_3 binding in dRLH-84 cells than in GH3 cells at an early phase of the nuclear uptake process and after binding equilibrium. These data suggest that SK&F L-94901 is more effectively transported to nuclear TRs in hepatic cells than in pituitary cells and therefore shows liver-selective thyromimetic activity. In conclusion, SK&F L-94901 discriminates hepatic cells and pituitary cells at the nuclear transport process. The cellular transporters responsible for this discrimination were not evident.

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Introduction

Thyroid hormones exert biological effects mainly through nuclear receptors by regulating the expression of hormone-responsive genes (Oppenheimer *et al.* 1987). However, present understanding of the mechanisms of hormone action is not sufficient to explain the diversity of thyroid hormone action in various tissues and at various levels of development. Studies have revealed that, even in the nucleus, models of hormone action are not simple but involve interaction of multiple forms of receptors with multiple nuclear proteins, and that thyroid-hormone responsive elements (TREs) located on the hormone-responsive genes are diverse (Baniahmad *et al.* 1993, Lazar 1993, Lopez *et al.* 1993, Miyamoto *et al.* 1993, 1997, Lee *et al.* 1995). Besides these nuclear events, many events occur before the hormones reach nuclear receptors. The hormones enter the cells through the plasma membrane, are distributed to various cellular compartments, are metabolized to inactive or active compounds and finally enter nuclei through the nuclear membrane. Cellular transport of the hormones could be by passive diffusion (Lein & Dowben 1961, Weisiger *et al.* 1992) but there is now evidence to support an energy-dependent carrier-

mediated process for the cellular uptake of thyroid hormones (Eckel *et al.* 1979, Krenning *et al.* 1989). Nuclear transfer of the hormones may also involve active processes (Oppenheimer & Schwartz 1985) and may be regulated independently (Ichikawa *et al.* 1992). Cellular transport of thyroid hormones shows analog specificity and stereospecificity (Oppenheimer & Schwartz 1985); furthermore, the cellular transport of different thyronine analogs involves different mechanisms and these diversities vary from organ to organ (Everts *et al.* 1996, Nagasawa *et al.* 1995). It is therefore possible that extranuclear events contribute to the diversity of thyroid hormone action in various tissues and at various levels of development.

SK&F L-94901 is a thyromimetic compound that exerts thyromimetic activity on the liver but not on the heart and pituitary gland when administered to rats *in vivo* (Underwood *et al.* 1986). We have examined how this compound exerts liver-selective thyromimetic activity and found that it discriminates different cell types at the level of the nuclear transport process, either by favoring nuclear uptake in the liver or by impairing nuclear uptake in the pituitary. This further verifies the presence of distinct mechanisms of transport of thyroid hormones into the nucleus.

Materials and Methods

Cultured rat hepatoma dRLH-84 cells (Hashizume *et al.* 1991, Tanaka *et al.* 1989), were provided by the Japanese Cancer Research Resources Bank (Tokyo). A continuous line of cultured cells derived from rat pituitary tumor GH3 cells (Tashjian *et al.* 1968) were obtained from the American Type Culture Collection (Rockville, MD, USA). Culture media, penicillin, streptomycin, trypsin and EDTA were from Flow Laboratories (McLean, VA, USA); fetal calf serum (FCS) was from Filtron (Brooklyn, Australia). [125 I]3,5,3'-tri-iodo-L-thyronine (L-T₃) (2800–3400 μ Ci/ μ g) and [125 I]thyroxine (L-T₄) (1080–1320 μ Ci/ μ g) were from New England Nuclear (Boston, MA, USA) and L-T₃ and L-T₄ were from Sigma Chemical Co. (St Louis, MO, USA).

Binding of thyronine analogs to isolated nuclei

The cells were harvested by trypsinization. Isolation of nuclei and nuclear [125 I]T₃ binding assays were carried out as described previously (Ichikawa *et al.* 1992). Phase-contrast microscopy revealed that the nuclear preparation was free of intact cells and had little cytoplasmic contamination and that integrity of the nuclei was well preserved; however, nuclei isolated in this way exhibited a separation of the outer nuclear membrane from the inner membrane on electron microscopy (Ichikawa *et al.* 1986). To determine the binding of SK&F L-94901 to the nuclear receptor, isolated nuclei were incubated with 14.4 pM [125 I]T₃ with various concentrations of SK&F L-94901, T₃ or T₄. The amount of SK&F L-94901 required to prevent [125 I]T₃ binding was used to estimate the potency of binding of SK&F L-94901 to nuclear receptors.

Preparation of soluble thyroid hormone receptor (TR) subtypes α 1 and β 1 using Sf9 insect cell expression

The methods used for construction of the human TR α 1 and β 1 transfer vectors, expression of recombinant TRs in Sf9 insect cells, extraction of recombinant proteins, and characterization of expressed receptors have been described previously (Miyamoto *et al.* 1997). In brief, the entire coding region of human TR α 1 cDNA in pMe21 (Nakai *et al.* 1988) was PCR-amplified and inserted into the BamHI–HindIII site of pBlueBacHisC (Invitrogen, San Diego, CA, USA). This plasmid was designed to produce 6 $\times$ (His) fused human TR α 1. For TR β 1, a 1.5 kb BamHI–EcoRI fragment containing human TR β 1 was inserted into the BglII–EcoRI cleaved baculovirus transfer vector pVL1392 (Invitrogen). This transfer vector was expected to produce a full-length non-fused human TR β 1 protein. General baculovirus procedures (insect cell culture, transfection, isolation and purification of recombinant plaques, infection) were performed as described previously (Summers & Smith 1988). Sf9 cells were

cultured in Grace's insect medium containing 10% FCS at 27 °C, as attached cells in flasks. Antibiotics (50 mg/ml gentamicin and 25 mg/ml fungizone) were used during protein expression and production of viral stocks. For the extraction of recombinant proteins, cells infected with recombinant baculovirus were harvested 72 h after infection by centrifugation at 800 $\times$ g for 10 min and washed twice with PBS. The washed cell pellet was resuspended in 20 mM Tris–HCl pH 7.5, containing 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride and 20% glycerol, and disrupted by three freeze–thaw cycles. Supernatants containing recombinant receptors were used for further experiments.

Activation of TR subtypes in COS1 cells

For determinations of the potency of compounds to activate TR α 1 or β 1, COS1 cells were co-transfected with pCDM designed to express TR α 1 or β 1 (Miyamoto *et al.* 1997) and PAL-TK-luciferase reporter plasmid containing two copies of the palindromic TRE sequence, 5'-GGATCCAGGTCATGACCTGGATCC-3' (Miyamoto *et al.* 1997). A calcium phosphate precipitation technique was used for the transfection. Twenty hours after transfection, transfected cells were incubated with various compounds for 24 h in culture media containing 10% FCS and cells were harvested for the determination of luciferase activity. Details of the methods of plasmid construction, transfection, cell culture and luciferase assay have been described elsewhere (Miyamoto *et al.* 1997).

Thyroid hormone uptake study

The cellular and nuclear thyroid hormone uptake study was performed as described previously (Ichikawa *et al.* 1992, Nagasawa *et al.* 1995). In brief, cells grown in 60 mm diameter plastic Petri dishes were used. After the cells reached the late logarithmic phase of growth, the growth media were replaced with fresh media containing FCS that was pretreated with resin (Samuels *et al.* 1979) to remove endogenous thyroid hormones. Exchange of the hormone-free media was performed at 48 h with a single exchange at 24 h before the experiment. Thirty minutes before the experiment, cells on Petri dishes were washed twice with Hanks' Balanced Salt Solution (HBSS) in order to remove FCS. Uptake studies were performed at 37 °C in 60 mm diameter plastic Petri dishes with 2 ml HBSS without serum. [125 I]T₃ (14.4 pM) with or without unlabeled thyronines was used. After the incubation, cells were immediately cooled on ice and washed twice with ice-cold HBSS. Subsequent procedures were performed at 0–4 °C. After the HBSS was aspirated, cells were lysed by a 10-min incubation with 2 ml 0.25 M sucrose, 1 mM MgCl₂ and 20 mM Tris–HCl, pH 7.4 (SMT solution) containing 0.5% Triton X-100. Cell lysate from one dish

Figure 1 Relative binding potency of various thyronine analogs to isolated nuclei from cultured rat hepatoma (dRLH-84) and pituitary tumor (GH3) cells. Binding of [125 I] T_3 to dRLH-84 cell nuclei or GH3 cell nuclei was determined in the presence of various concentrations of iodothyronine analogs (T_3 , T_4 and SK&F L-94901). Data are means $\pm$ S.D. of three determinations from one experiment; similar results were obtained in a second experiment.

was divided into two 0.8 ml aliquots in glass tubes. The radioactivity of one aliquot was measured for the determination of whole-cell thyroid hormone uptake. The other aliquot was centrifuged at $1500 \times g$ for 10 min at 4°C . The resultant nuclear pellet was washed twice with SMT and the radioactivity of the nuclear pellet measured for determination of nuclear thyroid hormone uptake. After the determination of the radioactivity in the nuclear pellet, sucrose was removed by two washes with ice-cold PBS, followed by two washes with ice-cold 0.4 M perchloric acid to determine the DNA content of the nuclear pellet in every tube (Burton 1956).

Results

SK&F L-94901 does not discriminate different TR subtypes

SK&F L-94901 inhibited [125 I] T_3 binding to isolated nuclei almost equally in GH3 cells and in dRLH-84 cells (Fig. 1), suggesting that this compound did not discriminate differences in TRs between these different cell types. Time courses of [125 I] T_3 binding to isolated nuclei in the absence or presence of SK&F L-94901 were indistinguishable between these two cell lines (data not shown). Using soluble TR α 1 and β 1 expressed in insect cells, we tested whether SK&F L-94901 discriminates these different TR subtypes. The results indicate that the binding affinity for SK&F L-94901 was almost identical between TR α 1 and β 1 (Fig. 2). In addition, when COS1 cells were co-transfected with plasmid expressing luciferase under the control of TRE (PAL-TK-luciferase) and with plasmid expressing either TR α 1 or β 1, SK&F L-94901 activated luciferase activity almost equally in both types of receptor (Fig. 3). As the experiments were carried out in media with FCS, which contains thyronine binding proteins,

Figure 2 Relative binding potency of various thyronine analogs to TRs α 1 and β 1. Binding of [125 I] T_3 to TR α 1 or β 1 was determined in the presence of various concentrations of iodothyronine analogs (T_3 , T_4 and SK&F L-94901). Data are means $\pm$ S.D. of three determinations from one experiment; similar results were obtained in a second experiment.

concentrations of thyronine analogs required for the activation of TRs were greater than those required for binding to TR in cultured cells (Figs 4–6) that were examined in the serum-free media. Neither COS1 cells transfected with PAL-TK-luciferase and pCDM without TR nor those with pCDM expressing TRs and TK-luciferase

Figure 3 Effect of thyronine analogs on PAL-TK-luciferase activity in COS1 cells co-expressing TR α 1 or TR β 1. COS1 cells co-transfected with plasmids containing PAL-TK-luciferase and plasmids expressing TR α 1 (upper panel) or TR β 1 (lower panel) were incubated for 24 h in culture media containing 10% FCS with various concentrations of T_3 , 3,5,3'-tri-iodo-L-thyroacetic acid (Triac) or SK&F L-94901 and luciferase activity was measured. The abscissa indicates the concentration of the thyronine analogs added to the media containing 10% FCS. ■, Luciferase activity without thyronine analogs. Data are means $\pm$ ranges of duplicate determinations from one experiment; similar results were obtained from a second experiment.

Figure 4 Time course of uptake of [125 I] T_3 by rat pituitary (GH3; upper panels) and hepatic (dRLH-84; lower panels) cells and nuclei. $\circ$, $\square$, Uptake values in the absence of SK&F L-94901; $\bullet$, $\blacksquare$, values in the presence of 10^{-7} M (GH3 cells) or 10^{-8} M (dRLH-84 cells) SK&F L-94901. Means $\pm$ ranges of duplicate determinations from one experiment are shown; similar results were obtained from a second experiment.

without PAL (the palindromic TRE) responded to thyroid hormones (data not shown).

Kinetic analysis of cellular uptake of T_3

The time course of cellular and nuclear uptake of [125 I] T_3 by intact cells was examined using cultured rat hepatoma cells (dRLH-84) and cultured rat pituitary tumor cells (GH3) in the absence or presence of SK&F L-94901 (Fig. 4). Concentrations of 10^{-7} M and 10^{-8} M SK&F L-94901 were used for GH3 and dRLH-84 cells respectively. Cellular uptake of T_3 over time was linear up to 5 min in both cells. We used the terms 'initial uptake' for 3 min incubation, 'early uptake' for 10 min incubation and 'binding equilibrium' for 120 min incubation. In GH3 cells, 10^{-7} M SK&F L-94901 inhibited cellular uptake of T_3 slightly and nuclear uptake of T_3 to about 40%. In contrast, in dRLH-84 cells 10^{-8} M SK&F L-94901 reduced nuclear T_3 uptake to 25–30% without affecting cellular T_3 uptake. These effects were seen consistently at every time point. The initial velocity (V_o) of cellular

uptake of T_3 was saturable. The double-reciprocal plot of V_o for cellular uptake of T_3 against T_3 concentration (Lineweaver–Burke plot) was linear at T_3 concentrations of 10^{-9} – 2×10^{-8} M (low K_m system) and 10^{-7} – 2×10^{-6} M (high K_m system). The Michaelis constant (K_m) and maximal velocity (V_{max}) values are given in Table 1. Cellular uptake of T_3 at low T_3 concentrations was inhibited competitively by high concentrations of SK&F L-94901 with K_i values of 130 nM and 1800 nM in GH3 and dRLH-84 cells respectively.

SK&F L-94901 inhibits hepatic nuclear uptake of T_3 more efficiently than pituitary nuclear uptake of T_3 in intact cells

In order to compare the nuclear access of SK&F L-94901 in pituitary (GH3) cells and hepatic (dRLH-84) cells, the potency of SK&F L-94901 to inhibit early nuclear uptake of [125 I] T_3 was examined using intact cells. Whereas approximately 73 nM SK&F L-94901 was required for half-maximal inhibition of GH3 nuclear uptake of [125 I] T_3 , as little as about 2.4 nM was sufficient to exert

Figure 5 Effect of unlabeled T_3 or SK&F L-94901 on initial uptake of $[^{125}I]T_3$ in rat cultured pituitary and hepatic cells. Initial uptake of $[^{125}I]T_3$ to cultured rat pituitary tumor (GH3; upper panel) and hepatoma (dRLH-84; lower panel) cells and to the respective cell nuclei was determined in the presence of various concentrations of T_3 or SK&F L-94901. Each value was divided by that obtained without unlabeled analogs and expressed on the ordinate. Data are means $\pm$ S.D. of three separate experiments, each performed using duplicate dishes.

half-maximal inhibition of dRLH-84 cell nuclear uptake of $[^{125}I]T_3$ (Fig. 5). These data indicate that SK&F L-94901 enters hepatic cell nuclei more efficiently than pituitary cell nuclei when intact cells are used. However when cellular entry was compared in these cells, the potency of SK&F L-94901 to inhibit cellular entry of $[^{125}I]T_3$ was more potent in GH3 cells than in dRLH-84 cells. As a result of the preferential entry of SK&F L-94901 to hepatic nuclei in intact cells, the inhibition by SK&F L-94901 of hepatic nuclear $[^{125}I]T_3$ binding was stronger at binding equilibrium compared with that of pituitary cell nuclear binding when intact cells were incubated with ligand for 2 h (Fig. 6). The results are compatible with the idea that nuclear exit of SK&F L-94901 does not contribute to liver-selective activity and that the liver-selective thyromimetic activity of SK&F L-94901 is due to preferential nuclear access.

We also analysed the ratios of nuclear to medium concentrations of SK&F L-94901 in these cells. The concentration of SK&F L-94901 required to inhibit nuclear $[^{125}I]T_3$ binding was about 480- and 23-fold greater than the ID_{50} of T_3 in whole-cell incubations of GH3 and dRLH-84 cells respectively (Fig. 6), compared with 264- and 380-fold values in the case of isolated nuclei (Fig. 1). Taking into account that T_3 is actively transported from medium to nucleus and that the free T_3 concentration is 5–10-fold greater in the nucleus than in media (Freake *et al.* 1986), nuclear free SK&F L-94901 concen-

Figure 6 Effect of unlabeled T_3 or SK&F L-94901 on cellular and nuclear $[^{125}I]T_3$ after binding equilibrium. Cultured rat pituitary tumor (GH3; upper panel) and hepatoma (dRLH-84; lower panel) cells were incubated with $[^{125}I]T_3$ and various concentrations of T_3 or SK&F L-94901. After 2 h of incubation, cellular $[^{125}I]T_3$ and nuclear $[^{125}I]T_3$ were determined. Each value was divided by that obtained without unlabeled analogs and expressed on the ordinate. Data are means $\pm$ S.D. of three separate experiments, each performed using duplicate dishes.

trations were 2.8–5.5 and 80–170 times greater than the free SK&F L-94901 concentrations in the media in GH3 and dRLH-84 cells respectively.

Discussion

In the present study, we studied the mechanisms by which SK&F L-94901 shows liver-selective thyromimetic activity. As the TRs exist in multiple forms such as $\alpha 1$, $\beta 1$ and $\beta 2$ and various organs contain them in different ratios (Schwartz *et al.* 1992), we tested whether SK&F L-94901 specifically recognizes the hepatic receptor or not. Our results showed that SK&F L-94901 bound equally to both hepatic and pituitary receptors in isolated nuclei. In

Table 1 Kinetics of cellular uptake of T_3 . K_m and V_{max} were calculated from double-reciprocal plots of T_3 concentration against initial (3-min) T_3 uptake by GH3 and dRLH-84 cells

	GH3 cells	dRLH-84 cells
Low K_m system		
K_m (nmol/l)	18	11
V_{max} (fmol/10 μ g DNA/min)	90	180
High K_m system		
K_m (nmol/l)	600	1000
V_{max} (fmol/10 μ g DNA/min)	2600	3900

addition, it bound and activated TRs $\alpha 1$ and $\beta 1$ almost equally. We therefore considered that the liver selectivity of SK&F L-94901 was not exerted at the receptor level. Instead, whole-cell incubation studies revealed that binding of SK&F L-94901 to the nuclear TR was significantly stronger in hepatic cells than in pituitary cells and that the binding of SK&F L-94901 to the receptor in whole cells was stronger than that in isolated nuclei in both cell lines. These findings were consistently observed throughout the time-course of the experiment from the initial phase of the uptake process to binding equilibrium. These data indicate that nuclear transport of SK&F L-94901 is active and that the nuclear concentration of SK&F L-94901 is greater than the extracellular concentration at binding equilibrium in both cell lines. SK&F L-94901 is more actively transported to the nucleus and shows greater occupancy of the nuclear receptors in hepatic cells than in pituitary cells, therefore achieving liver-selective thyromimetic action. Other rat hepatoma cells (Hepa 1) and cultured human hepatoblastoma cells (Hep G2) gave results similar to those obtained with dRLH-84 cells, whereas cultured human fibroblasts showed results similar to those with GH3 cells (data not shown).

As our data concerned the inhibition of [125 I] T_3 binding by unlabeled SK&F L-94901, we do not know the cytoplasmic concentrations of SK&F L-94901. The less potent inhibition of dRLH-84 cellular uptake of [125 I] T_3 by SK&F L-94901 is relevant only to the uptake processes common to T_3 and SK&F L-94901. If there are alternative cellular processes of SK&F L-94901 uptake that are not shared by T_3 , we would not be able to detect them unless we use radiolabeled SK&F L-94901. However, our assay gives correct information on the binding of SK&F L-94901 to nuclear TR, as more than 98% of nuclear [125 I] T_3 was bound to nuclear TR, as verified by the binding specificities and relative binding affinities to various thyronines. We are therefore able to conclude that hepatic nuclei take up and concentrate more SK&F L-94901 than do pituitary nuclei, as a result either of enhanced cellular uptake or of enhanced transfer from cytoplasm to nucleus. It is also possible that instead of favoring the nuclear uptake of SK&F L-94901 in the liver, mechanisms may exist that impair the nuclear uptake of SK&F L-94901 in the pituitary.

What makes SK&F L-94901 preferentially enter hepatic nuclei in intact cells? We showed previously that rat hepatic cytosol contains thyroid hormone binding protein (cTBP) (Hashizume *et al.* 1991, Nishii *et al.* 1993, Takeda *et al.* 1994), the thyroid hormone binding of which is markedly stimulated by NADPH or NADP and DTT. Subsequent studies revealed that NADPH-activated cTBP inhibits but NADP-DTT-activated cTBP promotes thyroid hormone uptake to nuclei. cTBP was abundant in cultured rat hepatoma cells (dRLH-84) (Hashizume *et al.* 1991) but was absent from cultured rat pituitary tumor cells (GH3) (data not shown). We therefore considered the

possibility that SK&F L-94901 might bind more potently to NADP-DTT-activated cTBP than to NADPH-activated cTBP, thus allowing more preferential nuclear transition in hepatic cells. However, binding of SK&F L-94901 to these different types of cTBP was almost equal (data not shown), eliminating such a possibility.

To date, we remain unaware of the mechanisms by which SK&F L-94901 discriminates hepatic nuclear transport and pituitary nuclear transport. It is possible that nuclear transport carriers are different in these different cell types. Isolation of the cellular thyroid hormone transporter is now in progress.

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