Nontranscriptional modulation of intracellular Ca\(^{2+}\) signaling by ligand stimulated thyroid hormone receptor

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Thyroid hormone 3,5,3'-tri-iodothyronine (T\(_3\)) binds and activates thyroid hormone receptors (TRs).

Here, we present evidence for a nontranscriptional regulation of Ca\(^{2+}\) signaling by T\(_3\)-bound TRs. Treatment of Xenopus thyroid hormone receptor beta subtype A1 (xTR\(_{\beta A1}\)) expressing oocytes with T\(_3\) for 10 min increased inositol 1,4,5-trisphosphate (IP\(_3\))-mediated Ca\(^{2+}\) wave periodicity. Coexpression of TR\(_{\beta A1}\) with retinoid X receptor did not enhance regulation. Deletion of the DNA binding domain and the nuclear localization signal of the TR\(_{\beta A1}\) eliminated transcriptional activity but did not affect the ability to regulate Ca\(^{2+}\) signaling. T\(_3\)-bound TR\(_{R} A1\) regulation of Ca\(^{2+}\) signaling could be inhibited by ruthenium red treatment, suggesting that mitochondrial Ca\(^{2+}\) uptake was required for the mechanism of action. Both xTR\(_{\beta A1}\) and the homologous shortened form of rat TR\(_{\beta 1}\) (rTR\(_{\beta A1}\) F1) localized to the mitochondria and increased O\(_2\) consumption, whereas the full-length rat TR\(_{\beta 1}\) did neither. Furthermore, only T\(_3\)-bound xTR\(_{\beta A1}\) and rTR\(_{\beta A1}\) F1 affected Ca\(^{2+}\) wave activity. We conclude that T\(_3\)-bound mitochondrial targeted TRs acutely modulate IP\(_3\)-mediated Ca\(^{2+}\) signaling by increasing mitochondrial metabolism independently of transcriptional activity.

Introduction

Thyroid hormones are lipophilic ligands composed of two iodinated tyrosine residues that regulate cellular differentiation and development, cardiac function, and basal metabolism (Abbaticchio et al., 1981; Oppenheimer et al., 1987, 1994; Nagai et al., 1989; Kawahara et al., 1991; Soboll, 1993a; Ichikawa and Hashizume, 1995). Thyroid receptors (TRs) are classified as steroid hormone receptors and have genomic effects similar to other nuclear receptors such as glucocorticoid, estrogen, and androgen receptors. Two separate genes encode thyroid hormone receptors \(\alpha\) (TR\(_{\alpha}\)) and \(\beta\) (TR\(_{\beta}\)). Alternative splicing or the use of different promoters generates multiple isoforms including the \(\alpha\) (TR\(_{\alpha 1}\), TR\(_{\alpha 2}\)) and \(\beta\) (TR\(_{\beta 1}\), TR\(_{\beta 2}\)) subtypes (Lazar, 1993). Thyroid hormones have been shown to increase the number of mitochondria and to induce the expression of mitochondrial proteins encoded by both nuclear and mitochondrial genes (Das and Harris, 1991; Soboll, 1993a; Iglesias et al., 1995; Wrutniak et al., 1995; Meehan and Kennedy, 1997; Schonfeld et al., 1997). Isolated mitochondria from hyperthyroid cells exhibit enhanced substrate cycling and increased oxygen (O\(_2\)) consumption (Soboll, 1993b). Thyroid hormone also affects the mitochondrial membrane potential (\(\Delta \Psi\)) through the expression of mitochondrial proteins (Soboll, 1993a,b). Collectively, these long-term effects take days or weeks to manifest and are thought to be mediated by nuclear and mitochondrial transcriptional regulation.

Increasing evidence suggests that thyroid hormone exerts nontranscriptional effects on mitochondrial metabolism. Initial studies demonstrated that treatment of cells with 3,5,3'-tri-iodothyronine (T\(_3\)) results in a rapid increase in O\(_2\) consumption and ATP production in rat liver mitochondria (Sterling, 1980). These effects persisted in the presence of protein synthesis inhibitors, suggesting that the mechanism of action was non-
transcriptional. Sterling and coworkers (Sterling, 1980; Sterling and Brenner, 1995) additionally demonstrated that exposure of mitochondria to T_3, isolated from rat hepatocytes, increased both ATP production and O_2 consumption. Acute exposure of isolated mitochondria to thyroid hormone has also been reported to increase ATP production and to increase mitochondrial Ca^{2+} efflux (Sterling et al., 1980; Crespo-Armas and Mowbray, 1987; Soboll, 1993a). Mitochondrial localization of TRs was originally reported by Sterling and coworkers (Sterling, 1991). Later, Ardail et al. (1993) identified two high affinity T_3 binding proteins in rat liver mitochondria. Wrutniak et al. (1995) and Casas et al. (1999) reported the presence of a high affinity (∼43 kD) T_3 binding protein in rat liver mitochondrial matrix extracts, which was identified as an NH_2-terminus shortened form of rat TR_A1 (rTR_A1). The full-length form of the rat thyroid hormone receptor alpha subtype 1 (rTR_A1) is predominantly localized to the nucleus where it binds to DNA response elements and regulates transcriptional events (Wrutniak et al., 1995). Wrutniak (Wrutniak et al., 1995) suggested that the mitochondrial form of the rTR may be involved in mitochondrial transcriptional activity.

Intracellular Ca^{2+} signaling has been intimately linked to mitochondrial metabolism. Several dehydrogenases within the citric acid cycle are Ca^{2+} dependent (McCormack and Denton, 1989). Ca^{2+} uptake into the mitochondria is a passive process driven by the mitochondrial ΔΨ and occurs via the Ca^{2+} uniporter. Because of the low Ca^{2+} affinity of the uniporter, high cytosolic Ca^{2+} concentrations are required to cause significant mitochondrial Ca^{2+} uptake. Under physiological conditions, these concentrations only occur near an open ion channel pore. Consequently, close physical proximity between the ER and mitochondria is required for significant mitochondrial Ca^{2+} uptake (Rizzuto et al., 1998, 1999). Work from our laboratory also demonstrated that mitochondrial Ca^{2+} uptake itself modulated inositol 1,4,5-trisphosphate (IP_3)-Ca^{2+} release (Jouaville et al., 1995). Subsequently, Hajnoczky et al. (1995) demonstrated that IP_3-mediated Ca^{2+} oscillations efficiently stimulated mitochondrial metabolism. The local Ca^{2+} signaling between the ER and mitochondria has now been supported by many other investigators (Simpson and Russell, 1996; Hajnoczky et al., 1999; Szalai et al., 2000). Control of mitochondrial metabolism by matrix Ca^{2+} appears to be a fundamental mechanism whereby cells meet their energy requirements.

Xenopus laevis oocytes do not express detectable levels of endogenous TRs (Banker et al., 1991; Kawahara et al., 1991; Eliceiri and Brown, 1994). Induction of TR expression in Xenopus laevis occurs during the embryonic stages of development (Yaoita and Brown, 1990; Banker et al., 1991; Kawahara et al., 1991; Eliceiri and Brown, 1994). Consequently, Xenopus oocytes offer a unique model system to study the effects of thyroid hormones and their receptors on intracellular Ca^{2+} signaling and mitochondrial metabolism.

We present evidence demonstrating that thyroid hormone-activated TRs acutely regulate mitochondrial metabolism and, thereby, Ca^{2+} wave activity. Only expression of the NH_2-terminus–truncated forms of TR that target the mitochondria were effective at stimulating mitochondria. Transcriptionally inactive TRs were fully capable of modulating Ca^{2+} wave activity. These observations suggest an acute nontranscriptional pathway for modulation of intracellular Ca^{2+} signaling via thyroid hormone receptor-stimulated mitochondrial metabolism.

**Results**

T_3-stimulated TR_A1s modulate IP_3-mediated Ca^{2+} wave activity

Acute nongenomic effects of thyroid hormones occur within minutes of ligand treatment (Hummerich and Soboll, 1989). To examine the importance of TRs on the nongenomic modulation of intracellular Ca^{2+} signaling, stage VI Xenopus oocytes were injected with mRNA encoding the Xenopus thyroid hormone receptor betz subtype A1 (xTR_A1) as described previously (Camacho and Lechleiter, 2000). Expression of xTR_A1 was confirmed by Western blot analysis, 2–3 d after mRNA injection (Fig. 1). The Ca^{2+} indicator dye was injected into oocytes 30–45 min before confocal imaging. When oocytes were injected with IP_3, we observed repetitive Ca^{2+} wave activity with interwave periods of 6.62 ± 0.20 s (n = 70; Fig. 1). When xTR_A1 expressing oocytes were treated with T_3 10 min before IP_3 injection, the Ca^{2+} wave periodicity increased significantly to 8.40 ± 0.30 s (Fig. 1, a and c; n = 24, P < 0.0001 ANOVA single factor). Treatment of oocytes with T_3 by itself did not induce Ca^{2+} release and no detectable changes in basal intracellular Ca^{2+} concentrations were observed. Application of T_3 ligand to nonexpressing control oocytes, had no effect on the
Similarly, xTR also significantly higher than that of control oocytes expressing oocytes exposed to T3 (0.69 Ca2+/H9252 at comparable levels (Western blots below lanes 4–7). TRR antibody (MA1-215). xRXR/cognate ligands.

Figure 2. Transcriptional activity of TR\textsubscript{A1} requires xRXR, and both cognate ligands. Transcriptional activity was monitored with the TRE-reporter vector, pSEAP\textsubscript{(TRE)}. (a) Lanes 1 and 2 are negative (pSEAP) and positive (pSEAP\textsuperscript{(+)} vector controls. Oocytes expressing TR\textsubscript{A1} or TR\textsubscript{A1} plus xRXR\textsubscript{A1} were incubated with 100 nM T3 (lanes 3–5) plus 100 nM RA (lane 5) for 3 d. Cytosolic extracts from each group of oocytes was prepared and loaded onto a 10% SDS-PAGE at 2.5 oocytes equivalents per lane. SEAP was detected with the polyclonal rabbit anti–human SEAP antibody and an HRP-conjugated secondary antibody. The SP labeled arrow indicates SEAP immunoreactivity, which was present only in oocytes expressing TR\textsubscript{A1} and xRXR\textsubscript{A1} exposed to both T3 and RA. (b) Transcriptional activity of TR\textsubscript{A1} requires the pBOX within the DBD and the NLS. Oocytes expressing xRXR\textsubscript{A1} and TR\textsubscript{A1} plus RA show no SEAP immunoreactivity when incubated with T3 (lane 6) or T3 plus RA (lane 7). Western blot analysis shows that xRXR\textsubscript{A1}, TR\textsubscript{A1}, and xRXR\textsubscript{A1} plus NLS are expressed at comparable levels (Western blots below lanes 4–7). TR\textsubscript{A1} and xRXR\textsubscript{A1} plus NLS were detected with the monoclonal mouse anti–human TR\textsubscript{A1} antibody (MA1-215). xRXR\textsubscript{A1} was detected with a polyclonal rabbit anti–human RXR antibody (Sc-774).

Ca2+ interwave period (6.38 ± 0.34 s, n = 27, Fig. 1, a and c). Similarly, xTR\textsubscript{pA1} expressing oocytes without T3 treatment exhibited no change in Ca2+ wave periodicity (6.97 ± 0.24 s, n = 35). Peak Ca2+ wave amplitudes (ΔF/F) for xTR\textsubscript{pA1} expressing oocytes exposed to T3 (0.69 ± 0.04, n = 29) was also significantly higher than that of control oocytes exposed to T3 (0.55 ± 0.04, n = 20; P < 0.05, t test). We conclude from these data that T3-stimulated xTR\textsubscript{pA1} acutely modulates IP\textsubscript{3}-mediated Ca2+ wave activity and that both thyroid hormone and receptor expression are required for these effects.

Transcriptional activity of TR\textsubscript{A1} is undetectable in the absence of xRXR

Classically, activated thyroid hormone receptors heterodimerize to initiate transcription responses. Retinoid X receptor (RXR) is the most common dimerization partner that binds to the thyroid hormone response element (TRE; Leid et al., 1992; Bhat et al., 1994; Wong and Shi, 1995). To investigate the transcriptional activity of xTR\textsubscript{pA1}, we coinjected oocytes with xTR\textsubscript{pA1} mRNA and a plasmid reporting vector containing a TRE system with two direct repeats (DR4) upstream of the secreted placent al alkaline phosphatase (SEAP) gene (p-TRESEAP; CLONTECH Laboratories, Inc.). If the hormone receptor dimerizes and binds to the TRE enhancer, the oocyte expresses SEAP, which is secreted into the medium. mRNA-injected oocytes were continuously bathed in T3 (100 nM) for 3 d and the presence of SEAP was subsequently quantified by Western blot analysis and used as a marker for transcriptional activity. Using this TRE-reporting system, we observed no transcriptional activity in oocytes expressing the xTR\textsubscript{A1} protein by itself (Fig. 2 a, lane 3). However, when we coexpressed xRXR\textsubscript{A1} with xTR\textsubscript{pA1} and oocytes were incubated with T3 (100 nM) and 9-cis retinoic acid (RA; 100 nM) for 3 d, SEAP expression was significantly increased (Fig. 2 a, lane 5). Note that xTR\textsubscript{A1}/xRXR\textsubscript{A1} mediated transcription requires both ligands, T3 and RA (Fig. 2 a, lanes 4 and 5). These data indicate that stimulation of xTR\textsubscript{pA1} by T3 does not initiate detectable transcription in Xenopus oocytes.

Acute modulation of Ca2+ signaling does not require heterodimerization with RXR

To test whether heterodimerization of xTR\textsubscript{pA1} with xRXR\textsubscript{A1} affects the acute modulation of Ca2+ activity, we coinjected oocytes with both xRXR\textsubscript{A1} and xTR\textsubscript{pA1} mRNA and confirmed protein expression levels using Western analysis 2–3 d after injection of mRNA (Fig. 3 b). Oocytes were loaded with Ca2+ indicator dye and confocally imaged. Oocytes coexpressing xTR\textsubscript{pA1} and
xRXRs were initially exposed to both T3 (100 nM) and RA (100 nM) 10 min before injection with IP3 (~300 nM). The average Ca2+ interwave period for xTRα1-expressing oocytes was 6.58 ± 0.26 s (n = 67), whereas that of xRXRα/xTRα1 coexpressing oocytes was 6.72 ± 0.31 s (n = 82; Fig. 3, a and c). These values were not significantly different from each other (P = 0.22), but were both significantly larger than values in the control oocytes that exhibited an average Ca2+ interwave period of 5.90 ± 0.43 s (n = 55, ANOVA single factor, P < 0.0001; Fig. 3, a and c). We conclude that the xRXRα coexpression does not affect the ability of T3-bound xTRα1 to modulate Ca2+ signaling.

The DNA binding domain and NLS of TRβA1 are not required for acute affects on Ca2+ signaling

The ability of T3-bound xTRα1 to rapidly modulate Ca2+ activity suggested a nontranscriptional mechanism of action. Our strategy to test this hypothesis was to delete the DNA binding domain (DBD) and mutate the NLS from the thyroid hormone receptor and test whether the mutant receptors were (a) transcriptionally inactive and (b) still effective at modulating Ca2+ signaling. Oocytes were injected with the p-TRE-SEAP plasmid reporting vector. This reporting system requires heterodimerization of xRXRα and xTRα1 to transactivate the reporter gene (Fig. 2 a). Consequently, test oocytes were co-injected with xRXR mRNA with mRNA encoding either wild-type xTRβ1 (control), mutant xTRβ1 lacking the NLS (xTRβ1Δ-NLS), or the mutant lacking both the NLS and the pBox (xTRβ1ΔpBox-NLS). Once injected, oocytes were continuously bathed in T3 (100 nM) and RA (100 nM) for 3 d. Expression levels of xTRβ1 mutants and xRXRα groups were comparable to xTRβ1 and xRXRα groups (Fig. 2 b, bottom). Using the expression of SEAP as a marker for transcriptional activity, we confirmed that oocytes expressing the xTRβ1 mutants and xRXRα proteins were transcriptionally inactive, whereas oocytes expressing wild-type xTRβ1 and xRXRα proteins exhibited strong transcriptional activity (Fig. 2, lanes 7 and 5).

Subsequently, we tested whether the transcriptionally inactive xTRβ1 mutants could still acutely regulate Ca2+ signaling. Oocytes were injected with xTRβ1 mRNA or its mutants and protein expression levels were confirmed using Western analysis 2–3 d after injection (Fig. 4 c). Oocytes expressing xTRβ1 or the mutants were exposed to T3 (100 nM) 10 min before injection with IP3 (~300 nM). Ca2+ activity was confocally imaged, as described above. The average Ca2+ interwave period for the control group (water-injected oocytes) was 6.6 ± 0.20 s (n = 70), which was significantly shorter than that in the xTRβ1 expressing oocytes (8.40 ± 0.30 s, n = 40; ANOVA single factor, P < 0.0001; Fig. 4 b, d; Fig. 1). More importantly, regulation of the Ca2+ wave period in oocytes expressing either the single mutant xTRβ1Δ-NLS (9.6 ± 0.48 s, n = 24) or the double mutant, xTRβ1ΔpBox-NLS (8.4 ± 0.28 s, n = 24) was indistinguishable from oocytes expressing wild-type xTRβ1 (Fig. 4, b and d). We conclude from these data that neither the pBox nor the NLS of TRβA1 is required for acute regulation of Ca2+ signaling.

T3-bound TRβA1 appears to regulate Ca2+ signaling by increasing mitochondrial respiration

We reported previously that pyruvate/malate-energized mitochondria increase the amplitude and interwave period of IP3-induced Ca2+ waves in Xenopus oocytes ( Jouaville et al., 1995). These effects on Ca2+ wave activity were similar to those observed in TRβ1 overexpressing oocytes with acute T3 incubation (Fig. 1). Sterling and colleagues (Sterling, 1980) initially reported that T3 increases mitochondrial metabolism, particularly oxidative phosphorylation, in less than 30 min. Consequently, we hypothesized that the regulation of Ca2+ signaling by T3-activated xTRβ1 was mediated by its acute modulation of mitochondrial metabolism, which, in turn, increased mitochondrial Ca2+ uptake. Our strategy to test this hypothesis was threefold. First, we examined the effect of T3 on Δψ in TRβ1-expressing oocytes using the potential sensitive dye tetramethylrhodamine ethyl ester (TMRE). Oocytes were bathed in 200 nM TMRE for 5 min before imaging fluorescence with two-

Figure 4. The pBox and NLSs of TRβA1 are not required for the acute regulation of Ca2+ signaling. (a) Schematic figure depicting the position of the pBox deletion in the DBD and the NLS modification within TRβ1. (b) Spatial-temporal stack of IP3-induced Ca2+ wave activity in control oocytes compared with oocytes expressing TR mutants ΔpBox-NLS and Δ-NLS. Oocytes expressing the TR mutants were incubated with T3 (100 nM) 10–15 min before IP3 (~300 nM) injections. (c) Western blot analysis confirming comparable levels of protein expression for both wild-type and mutant TRβ1. (d) Histogram of the average Ca2+ wave periods for each group of oocytes (n values are in parentheses). Statistic significance over control oocytes is indicated by the asterisks (**; ANOVA single factor, P < 0.0001).
injected a subgroup of the TR/H9252
a polycation that inhibits the electrogenic mitochondrial Ca2+
were injected with buffer only. We found that Ru360 treatment
from a resting value of 0.33
0.0001) at 25 min (Fig. 5). These data suggest that T3-bound
log of mitochondrial TMRE fluorescence (Fmito) divided by the cytosolic fluo-
refers to the number of mitochondrion analyzed. Statistical significance is
indicated by the asterisks (***; ANOVA single factor, P < 0.001).

Figure 5. T3 stimulation of oocytes expressing TRaA1 increases the ΔΨ.
(a) Images of mitochondria labeled with the potential sensitive dye TMRE. The
oocytes are expressing TRaA1 and have been exposed to T3 for the
indicated amount of time. Images are 50 × 100 μm. (b) Histogram of the
of oocytes. Values in parentheses
indicated amount of time. Images are 50

photons were emitted from a relaxed state of individual mitochondria upon photon excitation (800 nm). ΔΨ was estimated by monitoring Log(Fmito/Fcyto) where Fmito is the fluorescence intensity of individual mitochondria and Fcyto is the cytosolic fluorescence (Farkas et al., 1989). We found that T3 significantly increased ΔΨ from a resting value of 0.33 ± 0.01 (n = 75) to 0.44 ± 0.01 (n = 92, P < 0.0001) at 5 min and to 0.48 ± 0.02 (n = 48, P < 0.0001) at 25 min (Fig. 5). These data suggest that T3-bound TRaA1 regulates Ca2+ signaling by increasing ΔΨ. Second, we injected a subgroup of the TRaA1-expressing oocytes with ruthenium 360 (Ru360; Calbiochem, 1 μM final concentration), a polycation that inhibits the electrogenic mitochondrial Ca2+ uniporter (Ying et al., 1991) ~60 min before IP3 injection and Ca2+ imaging. A control group of TRaA1-expressing oocytes were injected with buffer only. We found that Ru360 treatment
completely inhibited the affect of T3-bound TRaA1 on Ca2+ wave activity (Fig. 6). Untreated TRaA1 expressing oocytes exhibited the expected increase in wave periodicity (7.65 ± 0.4 s, n = 11) when preexposed to T3 for 10 min. However, the average wave period of Ru360-treated TRaA1 expressing oocytes was only 5.75 ± 0.22 s (n = 13) when preexposed to T3. The Ru360-treated average was nearly identical to untreated control oocytes (5.59 ± 0.04 s, n = 4) as well as Ru360-treated nonexpressing oocytes (5.53 ± 0.4 s, n = 3). These data are consistent with the hypothesis that T3-bound TRaA1 regulates Ca2+ signaling by increasing mitochondrial Ca2+ uptake via an increase in ΔΨ.

Third, we directly test whether thyroid hormone receptor together with T3 stimulates mitochondrial respiration. Xenopus oocytes were injected with TRaA1 mRNA or water and incubated for 3 d. The rate of O2 consumption was measured as an indicator of respiration. 200 oocytes in each group were loaded into a 2-ml O2 probe chamber filled with modified barth’s solution (MBS) solution. After 15 min of stabilization, the medium was exchanged with fresh MBS and O2 consumption was monitored for 30 min. The medium was exchanged a third time with MBS containing 100 nM T3 and O2 consumption was followed for another 30 min (Fig. 7, a and b). After this protocol, the rate of O2 consumption in water-injected oocytes after T3 exposure was 0.42 ± 0.25 nmol/min (n = 8). In contrast, the rate of O2 consumption in TRaA1-injected oocytes after T3 exposure was significantly increased to 1.68 ± 0.52 nmol/min (n = 4, P < 0.05). These data support the hypothesis that a T3/TRaA1-mediated increase in mitochondrial respiration was responsible for the modulation of IP3-mediated Ca2+ wave activity.

TRs targeted to the mitochondria are required for a T3-stimulated increase in respiration and the regulation of Ca2+ signaling
T3 treatment has previously been reported to increase mitochondrial metabolism (Sterling et al., 1980; Soboll, 1993a).

Figure 6. Ru360 blocks T3-bound TRaA1 increases in IP3-induced Ca2+ wave period. (a) Spatial-temporal stacks of the effect of Ru360 treatment on Ca2+ wave activity in control oocytes compared with oocytes expressing TRaA1 as labeled. (b) Histogram of average interwave period (seconds) of each group of oocytes shown in a. The asterisk (*) denotes a statistic significance using ANOVA single factor (P < 0.01). Values in parentheses represent the number of oocytes.

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Our data suggest that the acute effects of T₃ on mitochondrial metabolism are likely to be mediated by T₃-activated thyroid hormone receptors. A truncated form of rat TR₁ (rTR₁ΔF) has been shown to localize to mitochondria matrix (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Furthermore, the NH₂ terminus of the Xenopus TR₁A1 that we used throughout this work has a high homology to the NH₂ terminus of rTR₁ΔF (Fig. 8 a). Our strategy in this experiment was to test whether mitochondrial targeting of TRs was necessary to modulate Ca²⁺ signaling. First, we examined the cellular targeting of xTR₁A1, rTR₁, and rTR₁ΔF by injecting Xenopus oocytes with their respective mRNAs. After 3 d of expression, mitochondria were isolated by centrifugation. Whole oocyte extract (minus mitochondria) and mitochondrial extract from each group were subjected to immunoprecipitation using a TR antibody (MA1-215; Affinity BioReagents, Inc.). The immunocomplexes (TRs/MA1-215) were loaded onto a 10% SDS-PAGE gel for Western blot analysis. As shown in Fig. 8 c, only xTR₁A1 and rTR₁ΔF were detected in the mitochondrial extracts. Full-length rTR₁ did not localize to mitochondria. These results are consistent with previous reports (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Our next step was to compare the rate of O₂ consumption for oocytes expressing either rTR₁ or rTR₁ΔF (Fig. 9). Consistent with its mitochondrial targeting, the rate of O₂ consumption in rTR₁ΔF expressing oocytes after T₃ exposure was significantly increased 1.88 ± 0.35 nmol/min (n = 3, P < 0.05). In contrast, the rate of O₂ consumption in oocytes expressing the full-length rTR₁ was not significantly affected by T₃ exposure (−0.16 ± 0.55, n = 3). We conclude that mitochondrial targeting of TRs is required for a T₃ mediated increase in mitochondrial respiration.

Finally, we tested whether targeting of TRs to mitochondria was required to regulate Ca²⁺ signaling. As before, oocytes were injected with either full-length rTR₁ or NH₂ terminus–truncated rTR₁ΔF mRNAs. Protein expression levels were measured 2–3 d after injection (Fig. 10 b). Ca²⁺ activity was confocally imaged 10 min after treatment with T₃ (100 nM). We found that the average Ca²⁺ interwave period for rTR₁ΔF-injected oocytes was 8.8 ± 0.26 s (n = 24), which was significantly higher (ANOVA single factor, P < 0.01) than full-length rTR₁-expressing oocytes (7.9 ± 0.38 s, n = 22) and the water-injected control group (7.2 ± 0.24 s, n = 30; Fig. 10, a and c). Together, these data strongly indicate that the regulation of Ca²⁺ signaling by T₃-activated TRs requires their localization within mitochondria.
Discussion

In this work, we report that the acute exposure of oocytes expressing mitochnondrionally targeted TR to T3 regulates IP3-mediated Ca2+ wave activity. We observed a T3-bound TR induced increase in the Ca2+ wave period and amplitude. These changes in Ca2+ activity were similar to those observed in Xenopus oocytes when mitochondria were energized with respiratory chain substrates (Jouaville et al., 1995). In that report, the modulation of IP3-mediated Ca2+ release was due to an increase in mitochondrial Ca2+ uptake via an increase in the ΔΨ. Our current work is consistent with this model because we could inhibit the effects of T3-bound TRs by inhibiting mitochondrial Ca2+ uptake with Ru360. We also directly demonstrated that T3 exposure increased ΔΨ in oocytes expressing TRs. An increase in ΔΨ could be attributed to either a direct effect on electron transport or to a decrease in proton leak (Gunter and Pfeiffer, 1990; Gunter and Gunter, 1994; Gunter et al., 1998). The application of T3 to mitochondria has been reported to decrease proton leak in several preparations (Crespo-Armas and Mowbray, 1987; Soboll, 1993a). However, we found that T3 exposure increases O2 consumption in TR-expressing oocytes. An increase in the rate of O2 consumption is not consistent with a decrease in proton leak. Together, our data favors the conclusion that T3-bound TR regulates Ca2+ activity by increasing ΔΨ via an increase in proton pumping by the respiratory chain.

Application of thyroid hormones to mitochondria has long been known to increase metabolism (Sterling, 1980). Mitochondria were also known to be target organelles of T3 accumulation in cells (Sterling et al., 1984; Morel et al., 1996). However, a mitochondrial hormone receptor that mediated these effects has never been conclusively identified. Sterling (1986, 1991) initially suggested that the adenine nucleotide translocator (ANT) bound to T3 with high affinity. Romani et al. (1996) also suggested that thyroid hormone had its specific mitochondrial target site at the matrix side of ANT. They found that bongkrekic acid, a membrane-permeant inhibitor of ANT, blocked a thyroid hormone-induced release of Mg2+ from mitochondria. On the other hand, Wrutniak and coworkers (Wrutniak-Cabello et al., 2001) found no evidence demonstrating a direct interaction between ANT and T3. Our data indicate that ANT alone is not the thyroid hormone receptor that mediates the regulation of mitochondrial metabolism. Rather, our data reveal that a mitochondrial targeted TR is a required element of acute thyroid hormone regulation of metabolism. The use of Xenopus oocytes in these experiments was crucial in this determination because oocytes do not express endogenous TRs (Yaoita and Brown, 1990; Kawahara et al., 1991). The ubiquitous expression of endogenous TRs would have hidden this finding in earlier studies.

The ability of specific thyroid hormone receptors to target mitochondria has been demonstrated by other investigators. A truncated form of rat TR1 (rTR1ΔF) and not its full-length form, localized to the matrix of mitochondria (Ardail et al., 1993; Wrutniak et al., 1995; Casas et al., 1999). Our work corroborated these reports and further demonstrated that the xTRΔA1, which is highly similar to rTR1ΔF, targeted the mitochondria. Casas and coworkers (Casas et al., 1999) reported that mitochondrial activity was stimulated by overexpression of p43 (mitochondria-targeted, truncated-TR), which in turn, stimulated mitochondrial genome transcription of some enzyme units that played a role in the respiratory chain. The p43 protein had the same affinity to T3 as the full-length TR, to bind to the D-loop of two mt-TREs in the mitochondria, leading to mitochondrial protein synthesis (Casas et al., 1999). Their data suggested that p43 bound to mt-TREs as a homodimer because no RXR-isofrom in the mitochondrial extract was detected (Casas et al., 1999). Hadzic suggested that the NH2 terminus of TRs plays a role in TR-homodimerization in mitochondria (Hadzic et al., 1998). Together, these studies demonstrated that mitochondrial-targeted TRs could regulate mitochondrial metabolism by initiating transcription. However, our results cannot be accounted for by this mechanism of action. Specifically, transcriptionally inactive TR mutants modulated Ca2+ wave activity with the same efficacy as the wild type, xTRΔA1. We confirmed that xRXRα was required for xTRΔA1 to transactivate a reporter gene in our system, but more importantly, the presence of xRXR did not affect the ability of xTRΔA1 to modulate Ca2+ activity. Thus, we concluded that the mechanism by which T3-activated TRs regulate Ca2+ signaling cannot be attributed to transcription.
Nongenomic effects of various steroid receptors have been reported for mineralocorticoids (Moura and Worcel, 1984; Zhou and Bubien, 2001), glucocorticoids (Borski, 2000; Borski et al., 2002), gonadal steroids (Pietras and Szego, 1975; Wasserman et al., 1980; Lieberherr and Grosse, 1994; Guo et al., 2002a,b; Minshall et al., 2002), vitamin D3 (Sergeev and Rothen, 1995), and thyroid hormone (Hummerich and Sobell, 1989; Davis and Davis, 1996, 2002; Rojas et al., 2003). Most of these studies proposed the presence of specific membrane-bound receptors for nongenomic effects; however, specific receptors were not cloned or identified. For thyroid hormones in particular, Davis and Davis (2002) suggested that the mechanism of the nongenomic effects of thyroid hormone may not require TRs, and could involve actions of the hormone itself on signal transduction pathway via specific G protein–coupled protein. Recent work by Scanlan et al. (2004) identified an endogenous, rapid-acting derivative of thyroid hormone that is a potent agonist of the G protein-coupled trace amine receptor (TAR1). Activation of TAR1 increased cAMP production, which in turn, would active protein kinase A and phosphorylation of multiple proteins in cells. Our results do not exclude a potential role of second messenger systems in the mechanism of action of T3 on mitochondria. Rather, they demonstrate that classic TRs, those that have long been known to regulate gene transcription, will also acutely regulate mitochondrial activity when bound by T3. Stimulation is dependent on mitochondrial targeting of the TR, but not on its ability to initiate transcription. Together, these observations reveal a nontranscriptional pathway for modulation of intracellular Ca2+ signaling via T3/Tr transitioned mitochondrial metabolism.

The discovery of T3/Tr-regulated Ca2+ signaling is potentially important for several reasons. First, any process that acutely regulates intracellular Ca2+ release will impact the multitude of Ca2+-sensitive cellular processes ranging from contractility and secretion to proteolysis and cell death. Second, the ability of a steroid hormone to increase proton pumping provides a rapid method to increase metabolism in response to short-term energy requirements; for example, during increased neuronal activity or during a transient increase in muscle activity. Third, and potentially more importantly, a rapid increase in mitochondrial Ca2+ uptake could protect cells under conditions of stress. Mitochondria have long been recognized for their capacity to sequester large Ca2+ concentrations under pathological conditions (Gunter et al., 1994). The ability to transiently remove Ca2+ from the cytosol could be used to minimize tissue damage after stroke in neuronal tissue or to reduce the instability of cardiac cells after periods of hypoxia. Clearly, the identification of a mitochondrial receptor for thyroid hormone-induced increases in metabolism offers a new pharmacological target from which it will be possible to regulate a broad range of physiological and pathological processes.

Materials and methods

Expression vector construction

The coding fragments of rat TR1 cDNA, Xenopus RXRα, [a gift from R.M. Evans, The Howard Hughes Medical Institute, Chevy Chase, MD, and The Salk Institute for Biological Studies, La Jolla, CA] were amplified by PCR with a forward primer 5′-ccctgattccagctggaccgagcaagc-3′ (for rTr1), 5′-ccctgattccagctggaccgagcaagc-3′ (for XRXRα) containing a BamHI site, and a reverse primer, 5′-atacgagctagtgaagctgggcttctac-3′ (for XRXRα) containing a HindIII site. The PCR products were then subcloned into the Xenopus oocyte expression vector, pGEM-HeNot between the BamHI and HindIII sites (Camacho and Lechleiter, 1995). All restriction enzymes were purchased from Life Technologies. The truncated form of rTr1–rTr1ΔN was generated by PCR at the second ORF of rTr1 cDNA using a forward primer, 5′-ccctgattcagctggagctgggcttctac-3′ containing a BamHI site, and a reverse primer, 5′-atacgagctagtgaagctgggcttctac-3′ containing a HindIII site. The PCR product was placed into the pGEM-HeNot vector between the BamHI and HindIII sites.

Xenopus TrR1A1 was amplified by PCR with primers 5′-gatccatgct-ctggagctgggcttctac-3′ and 5′-atacgagctagtgaagctgggcttctac-3′ and subcloned into vector pGEM-HeNot between the BamHI and HindIII sites to create pGEM-HeNot–XTrR1A1. Xenopus mutant xTrR1A1–ΔNLS had its NLS removed by modifying the sequence from KR to AA. xTrR1A1–ΔBox-NLS had the same NLS modification as well as the pBOX deletion of CEGCK within the DBD. Both mutants were generated by QuikChange site-directed mutagenesis [Stratagene] using pGEM-HeNot–xTrR1A1 as a template. The forward primer for xTrR1A1–ΔNLS was 5′-gatccatgct-ctggagctgggcttctac-3′ and the reverse primer for xTrR1A1–ΔBox-NLS had the same NLS modification as well as the pBOX deletion of CEGCK within the DBD.

In vitro transcriptions and oocyte protocols

Synthetic mRNA was prepared as described previously (Camacho and Lechleiter, 1995). In brief, the pGEM-HeNot vector containing cDNA template was linearized by a Not restriction enzyme. From the linearized templates, mRNA was generated using the T7 promoter (MegaScript; Ambion). Cap analogue, m7G5′ppp(5″) (Ambion) was added to the reaction. The mRNA products were quantified by 1% agarose gel and spectrophotometry. RNase-free synthetic RNAs were resuspended at a concentration of 1.5–2.0 μg/μl and stored in aliquots of 3 μl at −80°C.

Stage VI oocytes were obtained from adult female Xenopus laevis. After defolliculation, oocytes were incubated in MBS (in mM: 88 NaCl, 1 KCl, 0.41 CaCl2, 0.33 Ca(NO3)2, 0.82 MgSO4, 2.40 NaHCO3, 10 Hepes, pH 7.5) at 18°C. mRNA was injected into the oocytes by a 50-nL bolus using a positive pressure injector [Nanoject; Drummond Scientific Co.]. Control oocytes were injected with diethyl pyrocarbonate-treated water. Oocytes were incubated at 18°C for 2–3 d to allow full expression of proteins in MBS supplemented with antibiotics streptomycin, penicillin, and fungizone [GIBCO-BRL]. Media was changed daily. Unhealthy oocytes were discarded daily.

Imaging acquisition and analysis

Ca2+ wave activity was imaged as described previously (Camacho and Lechleiter, 1995). In brief, oocytes were injected with 50 nl of a fluorescent Ca2+ sensitive dye (0.25 mM, Oregon green BAPTA2-cell impermeant; Molecular Probes) and incubated for 30–60 min before the experiment. Images were acquired with a confocal laser-scanning microscope (model PCM2000; Nikon) attached to an inverted microscope (model TE2000; Nikon) at the rate of 1.5 images/s. We used a 10× 0.45 NA objective (UVFLUOR; Nikon). Each group of mRNA-injected oocytes was randomly assigned into two subgroups, one was exposed to 100 nM of T3 for 10 min and the other was untreated with T3. Ca2+ wave activity was initiated by injecting a 50-nl bolus of 6 μM InP. The Ca2+ waves were analyzed with ANALYZE software [The Mayo Foundation, Rochester, MN]. Statistical significance was calculated by either one-factor ANOVA or a t test as indicated.

ΔΨ was estimated as described previously (Lin and Lechleiter, 2002). In brief, 200 nM TMRE (Molecular Probes) was added to the bath and images were acquired with a 60× 1.4 NA objective on the Nikon PCM2000 custom adapted for two-photon imaging. TMRE was excited at 800 nm using a Ti:Sapphire Coherent Mira 900 Laser pumped with a 3W Verdi laser (Coherent Inc.). Laser intensity was attenuated with a neutral-density filter wheel such that no detectable photobleaching of TMRE was observed.

Transcriptional activity assay

The transcriptional activity of TR and mutants were confirmed by using a reporting vector with the thyroid hormone response element (TRE) as a cis-acting en
hancer for the SEAP gene (Merck Pathway Profiling SEAP System; CONTECH Laboratories, Inc.). The negative control vector (pSEAPβgal) lacks the enhancer element, but contains a promoter and SEAP reporter gene. Oocytes in each group were injected with mRNA (0.5 μg) and vector (0.5 μg) as designated, and incubated in 1 ml MBS with 100 nM T3, and/or RA for 3 d. Media was exchanged and replaced every 24 h for 3 d. Collected media from each group was pooled, concentrated (Amicon ultra 10000 MWCO; Millipore) to 40 μl and run on a 10% SDS-PAGE. Oocyte cytosolic extract from each group was prepared and loaded onto 10% SDS-PAGE at amounts equivalent to 2.5 oocytes per lane. SEAP was detected with polyclonal rabbit anti–human SEAP antibody (Zymed Laboratories, Inc.). HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc.) was used and visualized by chemiluminescence (PerkinElmer).

Western blot analysis
Oocytes were washed twice times in homogenization buffer ([in mM]: 15 Tris-HCl, 140 NaCl, 250 sucrose, 1% Triton X-100, Complete protease inhibitor cocktail) at a concentration of 40 μl/oocyte. Washed oocytes were homogenized and centrifuged at 4,500 g for 15 min at 4°C. The supernatant was collected and loaded at 0.5 oocytes per lane onto 10% SDS-PAGE. TRs and mutants were detected with monoclonal mouse anti-human TRα antibody (MA1-215; Affinity BioReagents, Inc.). xRRX, was detected with polyclonal rabbit anti–human RXRs antibody (Sc-774; Santa Cruz Biotechnology, Inc.). HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc.) was used and visualized by chemiluminescence (PerkinElmer).

Cytosolic and mitochondrial extract preparations
300 oocytes in each group were washed twice times in homogenization buffer (in mM: 15 Tris-HCl, 140 NaCl, 250 sucrose, 1% Triton X-100, Complete protease inhibitor cocktail) at a concentration of 40 μl/oocyte. Washed oocytes were homogenized and centrifuged at 4,500 g for 15 min at 4°C. The supernatant was collected and loaded at 0.5 oocytes per lane onto 10% SDS-PAGE. TRs and mutants were detected with monoclonal mouse anti–human TRα antibody (MA1-215; Affinity BioReagents, Inc.). xRRX, was detected with polyclonal rabbit anti–human RXRs antibody (Sc-774; Santa Cruz Biotechnology, Inc.). HRP-conjugated secondary antibody (Jackson Immunoresearch Laboratories Inc.) was used and visualized by chemiluminescence (PerkinElmer).

O2 consumption assay
A biological O2 monitor (model 5300; YSI Inc.) was used to measure O2 consumption. 200 oocytes in each group were loaded into a 2 ml O2 probe chamber avoiding contact of the oocytes with the O2 probe. 1.5 ml of MBS was added to the chamber and the system was allowed to stabilize for 15 min. The medium was subsequently exchanged with 1.25 ml of fresh MBS solution and O2 consumption was monitored for 30 min. The media was exchanged again with MBS containing 100 nM T3 and O2 consumption was followed for the next 30 min. The slope of O2 levels was calculated before and after the addition of T3.

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