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Evidence for ADP-ribosylation in the mechanism of rapid thyroid hormone control of mitochondria

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Triiodothyronine in vitro at concentrations between 10^{-13} and 10^{-11}M very rapidly activates oxidative phosphorylation in hypothyroid rat liver mitochondria. Comparing the concentrations of hormone with estimates of the amounts of respiratory chain components present suggests that this activation may involve an amplification mechanism. Here we present evidence that while no changes in phosphorylation were detected following hormone administration, nicotinamide, an inhibitor of mono ADP-ribosylation reported to occur rapidly and reversibly in mitochondria, prevented activation by hormone. Moreover incubation with nicotinamide of euthyroid mitochondria and derived intact inner membrane vesicles revealed lowered ADP/O ratios under the same conditions as shown by hypothyroid preparations. While this lesion could be reversed simply by washing the intact mitochondria, the membrane vesicles required triiodothyronine addition.

Triiodothyronine; Oxidative phosphorylation; ADP-ribosylation; Covalent modification; Nicotinamide; Hypothyroidism

1. INTRODUCTION

Thyroid hormone has long been proposed to regulate mitochondrial metabolism and in recent years evidence has begun to accumulate that triiodothyronine (T_3), in addition to proposed longer term effects exerted through transcription and translation, can elicit a very rapid direct response from mitochondria [1]. A number of laboratories have shown that mitochondria acquire T_3 preferentially at very short times after administration in near-physiological amounts to thyroidectomised animals [2,4]. Saturable T_3 binding sites with K_a of 2×10^{-11} M^{-1} have been reported in inner mitochondrial membranes from a number of tissues which respond to thyroid hormone (e.g. liver, kidney, heart) and to be absent from those (e.g. adult brain, spleen and testis) which are unresponsive [5]. Two other groups have described similar binding sites in liver [6] and kidney [7] mitochondria and partial purification of a T_3 binding component (K_a 2-6 $\times$ 10^{11} M^{-1}) from beef heart mitochondria has recently been reported [8].

To complement these studies of a putative receptor, rapid effects of hormone on energy-driven mitochondrial processes have been observed both with isolated mitochondrial preparations [2,3,9-16] and with intact cells [17] and tissues [18-20]. One controversial aspect of the studies on isolated mitochondria is that while some laboratories have found that liver mitochondria from thyroidectomised rats show lowered ADP/O ratios with no evidence of uncoupling or loss of respiratory control [3,10,11,13,15] others, while finding different changes in oxidative phosphorylation in hypothyroid preparations which would be relatively rapidly altered by thyroid hor-

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mone, reported no change in the ADP/O ratio in their experiments [2,21]. We have recently been able to duplicate both these findings with the same mitochondrial preparation and to show that in order to observe a lowered ADP/O ratio with T_3-deficient mitochondria the presence in the buffer of Ca^{2+} is required but at free concentrations generally regarded as too low to influence mitochondrial function. As $[Ca^{2+}]$ free is raised to around 25 nM the ADP/O ratio is progressively lowered to its minimum value: raising the $[Ca^{2+}]$ further across the physiological resting-cell range, to levels below those which stimulate respiration, has no additional effect [16]. We have also found with hypothyroid mitochondria incubated in the presence of a buffered salt solution containing 25 nM Ca^{2+} that the lowered ADP/O ratio can be significantly raised by 10^{-13} M T_3 and maximally restored by 10^{-11} M hormone within 2 min of addition [16]. Since it can be calculated that the ATP synthetase/respiratory assembly content of our incubations is about the same as the free $[Ca^{2+}]$, it follows that T_3 must be exerting its effect by a mechanism capable of conferring around 100 times amplification in a short time. Because the most likely mechanism for a relatively long-lived stimulation of this sort would be covalent modification we have investigated this and report here that while we found no evidence for hormone-induced changes in protein phosphorylation, the stimulatory mechanism appears to involve mono ADP-ribosylation of inner membrane components.

2. MATERIALS AND METHODS

2.1. *Materials*

Adenosine 5′-[γ-^{32}P]triphosphate tetra (triethylammonium) salt (3×10^3 Ci/mmol) was purchased from NEN Research Products (England), nicotinamide from BDH (England) and digitonin from Kock-Light Ltd (England). All other materials were obtained as described in [16,22].

2.2. *Animals and mitochondria*

Male rats (130–150 g) of a Sprague-Dawley strain bred in the department were thyroidectomised and maintained on normal diet except that their drinking water contained 0.5 mM calcium lactate. Animals were used after at least 6 weeks when their weight was constant at 180–220 g (weight of the normal control liter mates > 350 g). Rats were killed by cervical dislocation and liver mitochondria prepared as described [14].

2.3. *Oxygen electrode experiments*

The ADP/O ratios were estimated [3] using 0.35 mg mitochondrial protein · ml^{-1} in oxygen electrode buffer (130 mM KCl; 2 mM $MgCl_2$, 2 mM EGTA, 5 mM Tris-HCl with and without 2% defatted albumin), pH 7.2, at 37°C in a Clarke-type electrode with 2 mM phosphate, 3.3 mM succinate as substrates in the presence of 3.3 $\mu g \cdot ml^{-1}$ rotenone and with 0.2 μmol ADP. Neither the ADP/O ratio nor state 4 respiration changed significantly with three successive additions of ADP to the same incubation. The free concentration of Ca^{2+} and EGTA was calculated as described in [16].

2.4. *^{32}P-phosphoprotein experiments*

Mitochondria were incubated (0.35 mg in 1 ml) as in the oxygen electrode experiments and brought to state 4 by the addition of 0.02 μmol ADP. To this suspension first 50 μl buffer containing 3.45 μCi [γ-^{32}P]ATP (3×10^3 Ci/mmol) and then after 2 min 3.3 μl of either buffer or T_3 (final concentration 10^{-9} M) were added and incubation continued for a further 2 min. The reaction was terminated by the addition of 330 μl of cold 50% (w/v) trichloroacetic acid and the mixture left for 30 min on ice before centrifuging. After 2 washes with 1 ml (10%, w/v) trichloroacetic acid and 3 washes with 1 ml ether, the dried protein pellet was dissolved overnight in PCS solvent and assayed for radioactivity as previously described [22].

2.5. *Mitoplast preparation*

Mitoplasts – intact mitochondrial inner membrane vesicles with the outer membrane and intermembrane space enzymes removed – were prepared by the controlled digitonin method of Schnaitman et al. [23] using 0.2 mg digitonin per mg mitochondrial protein followed by differential centrifugation as described by Schnaitman and Greenawalt [24]. The mitoplasts were finally resuspended in 0.25 M sucrose.

2.6. *Other methods*

These were as previously described [16,22].

3. RESULTS AND DISCUSSION

It has been demonstrated that the lowered ADP/O ratio shown by hypothyroid mitochondria in the external presence of 25 nM free Ca^{2+} could be completely restored in vitro within 2 min to values indistinguishable from euthyroid preparations by 10^{-11} M T_3 [16]. Hence it can be argued that if this were to involve a covalent modification this should be complete within 2 min.

To investigate the possibility that the mechanism involves protein phosphorylation, hypothyroid mitochondria in state 4 were incubated with high specific activity [γ-^{32}P]ATP to label the nucleotide pool and then treated with 10^{-9} M T_3 for 2 min. The washed denatured mitochondrial protein was then assayed for covalently bound ^{32}P-phosphate (for details see section 2). In two separate sets of experiments no difference was observed between label incorporated in the absence of T_3 and in the presence of T_3. The average radioactivity incorporated was 1314 dpm in the first experiment and 1586 dpm in the second. Given that the endogenous ATP content in these mitochondria is around 9 nmol/mg [12], the resulting specific radioactivity of the ATP in these incubations was around 300 dpm/pmol. Estimates of the content of F_1-ATPase put it at about 30 pmol/mg protein and the quantity of respiratory chain-linked proton pumps at about three times this [25]. Hence incorporation of 1 mol ^{32}P per mol F_1-ATPase in our preparations might have been expected to raise the protein-bound radioactivity by about 3000 dpm. That no change was detected with total radioactivity around 1500 dpm suggests that no appreciable change in [^{32}P]phosphate incorporation followed hormone addition.

Two groups [26,27] have recently shown that rat liver mitochondria possess an active ADP-ribosyltransferase or perhaps an NAD glycohydrolase followed by a surprisingly specific non-enzymatic ADP-ribosylation [28]. This activity mono ADP-ribosylates an inner membrane protein whose molecular mass is around 31 kDa on SDS-PAGE [27] and which shows rapid turnover of the covalent modification in vivo. Furthermore, the activity is strongly inhibited by 10 mM nicotinamide [27]. We thus tested the effect of adding nicotinamide to our hypothyroid mitochondria. Table 1 shows that while in the absence of nicotinamide, 10^{-10} M T_3 raised the ADP/O ratio lowered in the presence of 25 nM free Ca^{2+} as

Table 1
The effect of nicotinamide on the ADP/O ratios of euthyroid and T_3-restored hypothyroid mitochondrial preparations

	ADP/O ratio		
		Normal	
Conditions	Thyroidectomised mitochondria	Mitochondria	Mitoplasts
–	1.57 ± 0.02 (5)	1.78 ± 0.03 (4)	1.59 ± 0.04 (5)[d]
+ T_3	1.79 ± 0.03 (3)[a]	1.81 ± 0.03 (4)	1.85 ± 0.06 (3)[b]
+ 10 mM nicotinamide	1.61 ± 0.03 (3)	1.64 ± 0.03 (4)[b]	1.48 ± 0.04 (3)
+ T_3 + 10 mM nicotinamide	1.54 ± 0.04 (4)[c]	1.59 ± 0.05 (4)[b]	1.47 ± 0.14 (3)

The hypothyroid mitochondria were incubated in standard buffer (see section 2) containing 25 nM free Ca^{2+} while for the euthyroid preparations the buffer contained 2% defatted bovine serum albumin. T_3, nicotinamide or both were added 2 min before ADP. The final T_3 concentration used was 10^{-10} M in the absence and 10^{-8} M in the presence of albumin. The average respiratory control ratios were 3.8, 4.2 and 2.5 for hypothyroid mitochondria, euthyroid mitochondria and mitoplasts, respectively. In the absence of 25 nM free Ca^{2+}, the ADP/O ratio of these same hypothyroid mitochondria was 1.82 ± 0.02 (5). See also the text for further details. The values are means ± SE for the number of separate preparations shown and the following significant differences were found using the *t*-test with Bessel's correction for small numbers: [a]$P<0.0025$, [b]$P<0.025$ vs the appropriate controls; [c]$P<0.01$ vs no nicotinamide; [d]$P<0.0125$ vs mitochondria

reported [16]. Addition of inhibitor prevented this restoration: nicotinamide alone had no appreciable effect. An investigation of the concentration effectiveness of nicotinamide in preventing T_3 action showed that at 1 mM it was barely effective (96% control restoration) while at 2,3 and 6 mM it allowed 38%, 17% and no restoration, respectively).

If rapid thyroid hormone action on mitochondria does operate via a mono ADP-ribosylation and the essential difference between a euthyroid preparation and a hypothyroid one is the presence of hormone in the former, then it is to be expected that nicotinamide should have an effect on mitochondria from normal rats since it would prevent effective hormone action. When euthyroid rat liver mitochondria were tested in the oxygen electrode buffer containing 25 nM free Ca^{2+}, 10 mM nicotinamide lowered the ADP/O ratio with succinate plus rotenone from 1.77 ± 0.02 to 1.59 ± 0.03 in three separate experiments ($P<0.005$). Table 1 presents data from a similar experiment in which the electrode buffer contains defatted bovine serum albumin which by containing low concentrations of Ca^{2+} has been found to raise the free Ca^{2+} sufficiently to lower the ADP/O ratios of hypothyroid but not euthyroid mitochondria [16]. The albumin confers stability, possibly by adsorbing membrane damaging products of phospholipid breakdown, and is particularly useful with submitochondrial preparations (see below). The albumin also adsorbs T_3, raising its effective concentration in this system to around 10^{-8} M [15]. As table 1 shows, 10 mM nicotinamide given 2 min previously lowers the ADP/O ratio in these euthryoid mitochondria, whether in the presence of added T_3 or not, to values characteristic of hypothyroid preparations. In two cases, mitochondria after incubation with nicotinamide were cooled, centrifuged, resuspended in nicotinamide-free buffer and recentrifuged before being retested in the oxygen electrode. The mean ADP/O ratio was 1.87 showing that this effect of nicotinamide was reversible.

The rapid reversal of the nicotinamide inhibition was not unexpected since presumably the euthyroid preparations still contained tightly bound hormone [5–8]. The outer membrane in particular has been reported to contain substantial thyroid hormone binding sites [7,29] some of which may be related to plasma thyroxine binding pre-albumin [29]. Hence we sought, by removing the outher membrane with digitonin [23] and leaving intact inner membrane vesicles, to reduce the endogenous pool of hormone. Table 1 presents the findings with mitoplasts prepared thus from euthyroid animals. These show the lowered ADP/O ratio seen with hypothyroid preparations which moreover can be raised by T_3 addition in vitro: nicotinamide prevented this hormone action as it did with mitochondria from thyroidectomised animals.

4. COMMENT

We have found that addition of 10 mM nicotinamide to mitochondria from hypothyroid rats, while having no other obvious effect, abolishes the in vitro restoration of the ADP/O ratio produced by added T_3. This suggests that mitochondrial processes under rapid thyroid hormone control may be regulated by mono ADP-ribosylation. As a corrollary the addition of nicotinamide to euthryoid mitochondrial preparations lowers the ADP/O ratio as might be predicted if indeed this is regulated by such a covalent modification. This mechanism is all the more attractive given that mono ADP-ribosylation has been found to be catalysed mainly by prokaryotic enzymes (e.g. [27]) when one considers the suggested origins of mitochondria as symbiotic bacteria. Moreover we [13] and others [30,31] have found elevated levels of pyridine nucleotides in hypothyroid mitochondria (and conversely lowered levels in hyperthyroid preparations [30]) which may reflect reduced (increased) use of substrate by the ADP-ribosyl transferase.

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