Increase in the adenine nucleotide translocase content of duckling subsarcolemmal mitochondria during cold acclimation

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Abstract Intermyofibrillar and subsarcolemmal mitochondria were isolated from duckling gastrocnemius muscle. The adenine nucleotide translocase (ANT) content of subsarcolemmal mitochondria was found to be half of that present in intermyofibrillar mitochondria. In addition, cold acclimation resulted in a 1.7-fold increase in subsarcolemmal mitochondrial ANT content, with intermyofibrillar mitochondrial ANT remaining constant. This change in mitochondrial ANT content correlates with the previously reported cold-induced change in the sensitivity of mitochondria to palmitate-inhibited ATP synthesis [Roussel et al. (1998) FEBS Lett. 439, 258–262]. It is suggested that the mitochondrial ANT content enhances or reduces the fatty acid uncoupling activity in tissue, depending on the energetic state of mitochondria.

Key words: Adenine nucleotide translocase; Cold acclimation; Skeletal muscle; Mitochondrion

1. Introduction

Despite their lack of brown adipose tissue, some birds can develop regulatory non-shivering thermogenesis (NST) after prolonged exposure to cold (see [1,2] for review). This cold-induced NST is primarily of skeletal muscle origin [3] and associated in situ with mitochondrial defects in coupling ([4], see also [1]). This is in agreement with the loosely isolated mitochondria described in the skeletal muscle of cold-acclimated birds [5–8]. Fatty acids have been suggested to be involved [6,9,10] in such thermoregulatory uncoupling, which was also postulated in mammals [9,11–13]. However, such a thermogenic mechanism would lead to depressed mitochondrial ATP production and would in turn challenge all the ATP-consuming processes within muscle. We recently addressed this question in isolated mitochondria where it was shown that even when maximally uncoupled by fatty acids, muscle mitochondria of cold-acclimated ducklings were still able to synthesize ATP at half their maximal rate [8]. As reviewed in [1], this suggests that muscular ATP-consuming processes can be fuelled even if NST mechanisms are occurring at the mitochondrial level.

Originally proposed by Skulachev [14], adenine nucleotide translocase (ANT) is widely known for mediating part of the fatty acid uncoupling activity in heart, skeletal muscle, liver and kidney mitochondria of various mammalian species [9,15,16], as well as in duckling skeletal muscle mitochondria (see Figure 6 in [1]). This hypothesis has now received further experimental support by the use of reconstitution experiments [17], azido derivatives of fatty acids [18] and translocase-deficient yeast mutants [19]. The inhibitory effect of fatty acids on the translocase activity [20,21] also supports this thesis. The ANT-mediated uncoupling of oxidative phosphorylation by fatty acids is of particular importance in the understanding of the mitochondrial mechanism of avian muscle NST for three reasons. Firstly, one of the proposed avian NST mechanisms is based on fatty acid-enhanced mitochondrial loose-coupling in skeletal muscle [6]. Secondly, UCP-1 is not expressed in birds [22] and there is still no experimental evidence for the presence of mammalian-like UCP in avian skeletal muscle, namely UCP-2 and UCP-3 (see [1]). Thirdly, cold exposure produces a mobilization of fat deposits and an increased supply of fatty acids to skeletal muscles (see [1] for review), presumably associated with a higher level of non-esterified fatty acids in tissue [10]. It is worth noting that ANT-mediated thermogenic uncoupling has been found in muscle mitochondria from ground squirrels waking up from hibernation and correlated with the content of non-esterified fatty acids [9]. With this in mind, the sensitivity of ATP synthesis to fatty acid inhibition has been found to be much higher in subsarcolemmal mitochondria (SSM) than in intermyofibrillar mitochondria (IFM) and essentially affected by cold acclimation in the former mitochondrial population [8]. The question therefore arose as to what the reason is for the dependence of the mitochondrial ATP synthesis sensitivity to fatty acids on the mitochondrial populations. In the present report we show that, in duckling skeletal muscle mitochondria, the ANT content is lower in SSM than in IFM and it is greatly increased in SSM after cold acclimation. We also point out that the mitochondrial ANT content accounts quantitatively for the sensitivity of mitochondrial ATP synthesis to fatty acid inhibition.

2. Materials and methods

Male muscovy ducklings (*Cairina moschata* L., pedigree R31, In-

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Abbreviations: ANT, adenine nucleotide translocase; CA, cold-acclimated; CAT, carboxyatractyloside; IFM, intermyofibrillar mitochondria; NST, non-shivering thermogenesis; SSM, subsarcolemmal mitochondria; TN, thermoneutral control

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stitut National de la Recherche Agronomique, France) from a commercial stockbreeder (Ets Grimaud, France) were used. They were fed ad libitum with commercial mash (Genthon 5A) and had free access to water. The cold acclimation schedule described by Barré et al. [23] was used: from the age of 1 week the ducklings were caged for a period of 5 weeks at either 4°C (cold-acclimated, CA) or 25°C (thermoneutral control, TN) in a constant photoperiod (light/dark: 8/16). According to the Ethical Committee of the Centre National de la Recherche Scientifique (CNRS), the present cold acclimation schedule received the agreement of the French Department of Animal and Environmental Protection.

Muscle SSM and IFM populations were isolated from the red part of the gastrocnemius muscle by a differential centrifugation procedure as described previously [8]. Protein concentration was measured by the usual biuret method using serum albumin as standard. The respiration of isolated mitochondria (0.5 mg protein/ml) was determined at 25°C with a Clark oxygen electrode and a Gilson 5/6H polarograph. The respiratory medium contained 200 mM sucrose, 5 mM succinate (sodium salt), 5 μM rotenone, 5 mM KH_2PO_4, and 20 mM Tris–HCl, pH 7.4, with a final fatty acid-free bovine serum albumin concentration of 2 mg/ml (0.2% w/v).

The ANT content was determined by titrating the rate of state 3 respiration with increasing concentrations of carboxyatractyloside (CAT) [24]. Mitochondria were preincubated with CAT in the respiratory medium for 1 min before ADP (0.1 mM) was added to initiate state 3 respiration. The mitochondrial content of ANT was determined by the amount of CAT required to completely inhibit state 3 respiration.

CAT was from Boehringer; the other compounds were purchased from Sigma. Results are expressed as mean value ± S.E.M. Analysis of variance (ANOVA) and Student's *t*-test were used for statistical calculations.

3. Results and discussion

Titrations with CAT of skeletal muscle mitochondria from TN and CA ducklings are shown in Fig. 1. In both IFM and SSM, ~55–60% of total ANT sites were blocked by CAT before a 10% decrease in the respiratory rate was observed, with no differences between TN and CA mitochondria. Once respiration rate is converted into phosphorylation rate with the ADP/O ratio, the slope of the inhibitory region is a measure of the turnover number for the ANT [25]. Under our experimental conditions, control IFM formed adenine nucleotides per translocase protein twice as fast as the corresponding SSM population (317 ± 37 min^{-1} vs. 155 ± 22 min^{-1}, respectively), and no effect of cold acclimation was found (385 ± 68 min^{-1} and 132 ± 17 min^{-1} in IFM and SSM from CA ducklings, respectively).

The mitochondrial content of active ANT was determined by the amount of CAT required to reduce state 3 respiration to the state 4 respiratory rate (Fig. 1), when CAT presumably saturated the translocase sites [24]. In control ducklings, the ANT content of IFM was found to be twofold higher than that in corresponding SSM (Fig. 2). Interestingly, the present IFM ANT content is in the range of reported values for mammalian skeletal muscle mitochondria, 920–1800 pmol/mg protein with an average content of ~1270 ± 330 pmol/mg protein [26–30]. It should be stressed that these studies were performed on mitochondria isolated from skeletal muscle homogenate first digested by proteinase. Although this experimental protocol results in a mixture of mitochondrial populations, the mitochondrial protein content would be mostly related to the IFM population. Hence, the present IFM ANT content can be reasonably compared with those above from mammalian skeletal muscle. Unfortunately no value of the ANT content in the skeletal muscle SSM population has clearly appeared in the literature so far, although one report has shown that SSM displayed lower ADP translocase activities than IFM in rat skeletal muscle [31]. Nevertheless, as far as duckling skeletal muscle mitochondria are concerned, it can be inferred from the results herein that almost half of the five-fold greater ATP synthesis rate noted in IFM in our prior study [8] would be explained by the higher ANT content found in this mitochondrial population compared to SSM. The other half would imply functional differences between these two mitochondrial populations in their capacity to form ATP, including substrate translocation and oxidation, electron transport chain and mitochondrial ATPase activities, as well as translocase activity. Although this requires further investigations, the present data argue that the translocase may be important in determining a regional difference in the mitochondrial oxidative phosphorylation capacity within skeletal muscle, at least in duckling.

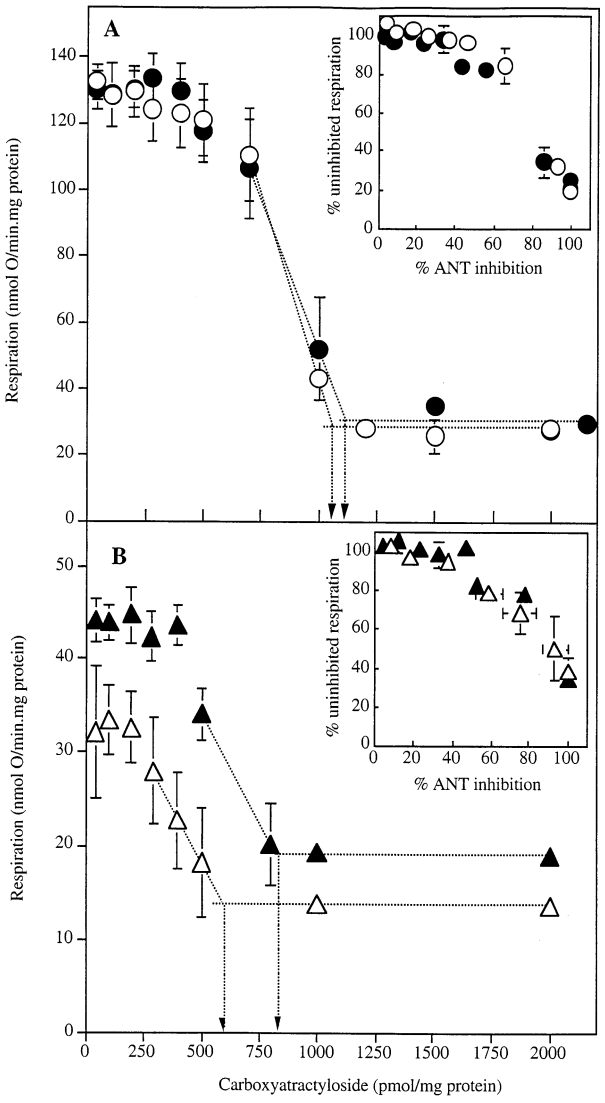

Fig. 1. Determination of ANT content by titration of active respiration with CAT. The IFM (A) and SSM (B) from thermoneutral (open symbols) and cold-acclimated ducklings (full symbols) were incubated with the respiratory medium, as described in Section 2. Inset: Threshold curves, percentage of uninhibited respiratory rate as a function of percentage of ANT inhibition by CAT. Each point comes from the corresponding experimental titration curve data.

The effect of cold acclimation on mitochondrial ANT content can be seen from the data shown in Fig. 2. The ANT content increased by 65% in CA SSM, while no significant change was noted in CA IFM. According to the threshold effect noted above, the higher SSM ANT content elicited by the cold acclimation will induce a 10% or more increase of the whole phosphorylation flux in this mitochondrial population. Although this could explain the higher SSM ATP synthesis rate previously found in CA ducklings compared to controls [8], it is not clear from the present study what was directly responsible for the 15% increase previously noted in IFM. It is therefore suggested that the biochemical mechanisms involved in the cold-increased ATP synthesis in IFM and SSM are most probably different. This hypothesis will require further research.

In the light of the present study, we wondered whether the mitochondrial ANT content noted therein can be related to the previously reported sensitivity of mitochondrial ATP synthesis to palmitate [8]. To test this hypothesis, the mean IC_{50} values given in [8] are plotted versus the corresponding mitochondrial ANT contents from both TN and CA ducklings. Fig. 3 shows that a linear relation is obtained between IC_{50} and ANT content. This demonstrates that the sensitivity of mitochondrial ATP synthesis to fatty acid-induced uncoupling depends on the mitochondrial ANT content, which extends Schönfeld's first observation on rat mitochondria [16]. However, while the ANT-mediated fatty acid-stimulated resting state respiration is enhanced as the mitochondrial ANT content is increased [16,32], our results also imply that the higher the mitochondrial ANT content, the less the mitochondrial ATP synthesis sensitive to fatty acid uncoupling. It thus appears that the ANT content would enhance or reduce the uncoupling effect of fatty acids in tissue, depending on the energetic state of mitochondria. Based on these observations, it is suggested that the ANT-mediated fatty acid uncoupling may be a mechanism of special interest for heat generation in skeletal muscle tissue at rest. Such thermoregulatory uncoupling would mostly take place in tissue containing ANT-rich mitochondria with low phosphorylation activity, namely resting skeletal muscle in a low ATP demand state. This hypothesis in turn implies that an increase of ATP demand within skeletal muscle (e.g. exercise) would reduce the activity of fatty acids as uncouplers. However, even if there is new evidence that fatty acids could act as uncouplers of cellular oxidative phosphorylation [33], the question whether non-esterified fatty acids are able to produce thermogenic uncoupling in tissues other than brown adipose tissue remains unclear and still awaits clear demonstration in muscles.

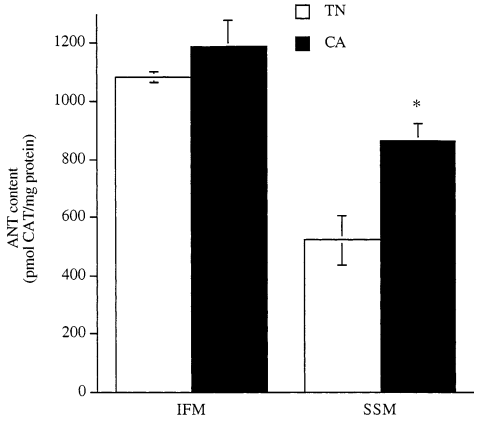

Fig. 2. ANT content of IFM and SSM from TN and CA ducklings. Values are means ± S.E.M. from 4–5 different preparations. The individual ANT contents were determined from the types of experiments as shown in Fig. 1. $*P < 0.05$, significant effect of cold exposure.

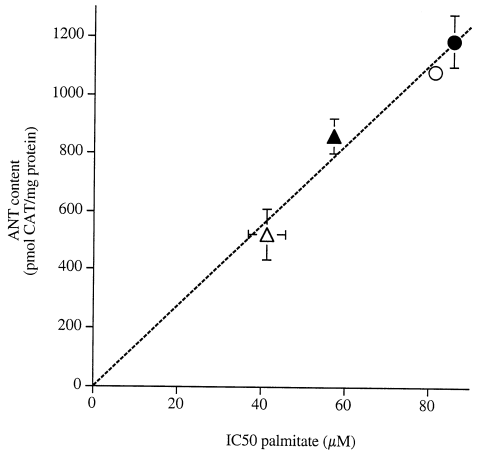

Fig. 3. Relationship between the palmitate sensitivity of mitochondrial ATP synthesis, as measured with the IC_{50} values reported in [8] to the mitochondrial ANT content from Fig. 2. The regression line equation is $y = 13.8x - 7.5$ ($r = 0.98$). Values are means ± S.E.M. Symbols: IFM (circles), SSM (triangles); TN (open symbols), CA (full symbols).

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References

[1] Duchamp, C., Marmonier, F., Denjean, F., Lachuer, J., Eldershaw, T.P.D., Rouanet, J.L., Morales, A., Meister, R., Bénistant, C., Roussel, D. and Barré, H. (1999) Ornis Fenn. 76, 151–165.
[2] Hissa, R. (1988) Acta Physiol. Scand. 132, 1–148.
[3] Duchamp, C. and Barré, H. (1993) Am. J. Physiol. 265, R1076–R1083.
[4] Duchamp, C., Cohen-Adad, F., Rouanet, J.L. and Barré, H. (1992) J. Physiol. 457, 27–45.
[5] Skulachev, V.P. and Maslov, S.P. (1960) Biochemistry (Moscow) 25, 1058–1064.
[6] Barré, H., Nedergaard, J. and Cannon, B. (1986) Comp. Biochem. Physiol. 85B, 343–348.
[7] Duchamp, C., Barré, H., Rouanet, J.L., Lanni, A., Cohen-Adad, F., Berne, G. and Brebion, P. (1991) Am. J. Physiol. 261, R1438–R1445.
[8] Roussel, D., Rouanet, J.L., Duchamp, C. and Barré, H. (1998) FEBS Lett. 439, 258–262.
[9] Brustovetsky, N.N., Egorova, M.V., Gnutov, D.Y., Gogvadze, V.G., Mokhova, E.N. and Skulachev, V.P. (1992) FEBS Lett. 305, 15–17.
[10] Levachev, M.M., Mishukova, E.A., Sivkova, V.G. and Skulachev, V.P. (1965) Biochemistry (Moscow) 30, 864–874.
[11] Skulachev, V.P., Maslov, S.P., Sivkova, V.G., Kalinichenko, L.P. and Maslova, G.M. (1963) Biochemistry (Moscow) 28, 54–60.
[12] Grav, H.J. and Blix, A.S. (1979) Science 204, 87–89.
[13] Herpin, P. and Barré, H. (1989) Comp. Biochem. Physiol. 92B, 59–65.
[14] Skulachev, V.P. (1991) FEBS Lett. 294, 158–162.

[15] Andreyev, A.Y., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.I. and Vygodina, T.V. (1989) Eur. J. Biochem. 182, 585–592.
[16] Schönfeld, P. (1990) FEBS Lett. 264, 246–248.
[17] Brustovetsky, N.N. and Klingenberg, M. (1994) J. Biol. Chem. 269, 27329–27336.
[18] Schönfeld, P., Jezek, P., Belyaeva, E.A., Borecky, J., Slyshenkov, V.S., Wiêckowski, M.R. and Wojtczak, L. (1996) Eur. J. Biochem. 240, 387–393.
[19] Polcic, P., Sabová, L. and Kolarov, J. (1997) FEBS Lett. 412, 207–210.
[20] Wojtczak, L. and Zaluska, H. (1967) Biochem. Biophys. Res. Commun. 28, 76–81.
[21] Wojtczak, L., Wiêckowski, M.R. and Schönfeld, P. (1998) Arch. Biochem. Biophys. 357, 76–84.
[22] Saarela, S., Keith, J.S., Hohtola, E. and Trayhurn, P. (1991) Comp. Biochem. Physiol. 100B, 45–49.
[23] Barré, H., Cohen-Adad, F., Duchamp, C. and Rouanet, J.L. (1986) J. Physiol. 375, 27–38.
[24] Vignais, P.V. (1976) Biochim. Biophys. Acta 456, 1–38.
[25] Forman, N.G. and Wilson, D.F. (1983) J. Biol. Chem. 258, 8649–8655.
[26] Willis, W.T. and Dallman, P.R. (1989) Am. J. Physiol. 257, C1080–C1085.
[27] Letellier, T., Malgat, M. and Mazat, J.P. (1993) Biochim. Biophys. Acta 1141, 58–64.
[28] Wisniewski, E., Kunz, W.S. and Gellerich, F.N. (1993) J. Biol. Chem. 268, 9343–9346.
[29] Roux, P., Le Saux, A., Fiore, C., Schwimmer, C., Dianoux, A.C., Trézéguet, V., Vignais, P.V., Lauquin, G.J.M. and Brandolin, G. (1996) Anal. Biochem. 234, 31–37.
[30] Rossignol, R., Letellier, T., Malgat, M., Rocher, C. and Mazat, J.P. (2000) Biochem. J. 347, 45–53.
[31] Joffe, M., Savage, N. and Isaacs, H. (1983) Comp. Biochem. Physiol. 76B, 783–787.
[32] Schönfeld, P., Wiêckowski, M.R. and Wojtczak, L. (1997) FEBS Lett. 416, 19–22.
[33] Hermesh, O., Kalderon, B. and Bar-Tana, J. (1998) J. Biol. Chem. 273, 3937–3942.