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Influence of non-esterified fatty acids on respiratory control of reconstituted cytochrome-*c* oxidase

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Bovine heart cytochrome-*c* oxidase was reconstituted in liposomes (asolectin) and the activity measured in the presence and absence of uncoupler at increasing concentrations of non-esterified fatty acids. Palmitic and stearic acids resulted in a decrease of about 40% in the respiratory control ratio at a concentration of 1 μM, when measured using a spectrophotometric procedure but not with a polarographic assay method. At higher fatty acid concentrations no further change was found. A 50% decrease in respiratory control was determined when the enzyme was reconstituted in pure phosphatidylcholine containing 2% cardiolipin. The respiratory control of reconstituted cytochrome-*c* oxidase from bovine liver was not influenced by fatty acids.

Cytochrome-*c* oxidase; Fatty acid; Respiratory control; Proteoliposome

1. INTRODUCTION

Fatty acids not only represent the main fuel for tissues consuming large amounts of energy, such as brown fat and heart, but also act as 'third messenger' for nonshivering thermogenesis in brown fat of newborn animals [1,2]. Nonshivering thermogenesis is turned on in brown fat tissue by the hormonal signal noradrenaline, causing lipolysis of fat stores via the second messenger cAMP. The concomitant increase in non-esterified fatty acids opens the proton channel of uncoupling protein [3] (thermogenin) in the inner mitochondrial membrane [1,4]. Respiration of brown adipocytes from cold-adapted guinea pigs increases about 10-fold after stimulation by noradrenaline [1]. Since no marked decrease in mitochondrial membrane potential of hormone-stimulated brown fat cells is found [1], non-esterified fatty acids may also be expected to stimulate the activity of cytochrome-*c* oxidase by direct interaction with the enzyme complex.

In a systematic study on nonshivering thermogenesis in Djungarian hamster the blood flow in various tissues was measured with respect to noradrenaline injection [5]. The hormone stimulated blood flow not only in brown fat tissue (10-fold stimulation in cold-adapted animals) but also in heart (3-fold). In contrast, no increase in blood flow was found in liver, brain, lung, or kidney, small intestine and skeletal muscle, where it decreased. The possible participation of the heart in nonshivering thermogenesis is of particular interest, since heart mitochondria do not contain uncoupling protein [1]. The heart could play a role in the thermal regulation of warm-blooded animals, like adult men, that do not have brown fat tissue.

Recently, Labonia et al. [6] described a partial uncoupling of reconstituted bovine heart cytochrome-*c* oxidase by micromolar concentrations of non-esterified fatty acids. Here, these results have been corroborated and extended. It is shown that in contrast to the bovine heart enzyme, the respiratory control ratio of reconstituted

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX, cytochrome-*c* oxidase

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bovine liver cytochrome-*c* oxidase is not decreased by non-esterified fatty acids.

2. MATERIALS AND METHODS

Asolectin (L-α-phosphatidylcholine, type IIS, from soybean), L-α-phosphatidylcholine (type VIIE from frozen egg yolk), cardiolipin (from bovine heart, sodium salt), cytochrome *c*, palmitic acid and stearic acid were obtained from Sigma (Munich). Asolectin was purified by the method of Kagawa and Racker [7]. Valinomycin and CCCP were purchased from Boehringer (Mannheim).

Cytochrome-*c* oxidase (COX) was prepared from bovine heart mitochondria by using cholate/deoxycholate [8,9] (cholate-COX). The enzyme from bovine liver and from bovine heart mitochondria was also prepared by employing Triton X-114/Triton X-100 as described in [10] (Triton-COX). The isolated enzymes were stored in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), at −80°C, and were reconstituted by the cholate-dialysis method [11] as described [12]. Alternatively, 39.2 mg phosphatidylcholine (type VIIE) and 0.8 mg cardiolipin (chloroform solutions) were evaporated to dryness and sonicated in 1 ml of 1.5% sodium cholate, 10 mM Hepes (pH 7.4), 40 mM KCl. Reconstitution of 3 μM COX was according to [12]. The orientation of the enzymes in the membrane was 70–80% right-side out, as determined by the method of Casey et al. [13].

COX activity was measured by the polarographic assay in 10 mM Hepes (pH 7.4), 40 mM KCl, 25 mM potassium ascorbate, 20 μM cytochrome *c* and proteoliposomes (25 nM cytochrome aa_3, final concentration) in the presence and absence of 1 μg/ml valinomycin and 3 μM CCCP [12]. Spectrophotometric assay of activity was performed in 10 mM Hepes (pH 7.4), 40 mM KCl, 10 μM ferrocytochrome *c* in the presence or absence of 1 μg/ml valinomycin and 3 μM CCCP as in [12]. Non-esterified fatty acids were added in alcoholic solutions (1 μl/ml final volume). 50 μl proteoliposomes were preincubated for 15 min at 0°C with 5 μl fatty acid solution or alcohol. The activity of COX is given as molecular turnover number (TN) in μmol 1/4 O_2·μmol cytochrome $aa_3^{-1}\cdot s^{-1}$ (polarographic assay) or in μmol ferrocytochrome *c*·μmol cytochrome $aa_3\cdot s^{-1}$ (spectrophotometric assay).

3. RESULTS

Bovine heart COX was isolated, using cholate and deoxycholate as solubilizing detergents, and reconstituted with asolectin in liposomes by the cholate-dialysis method. The activity of the enzyme was measured with the polarographic method at increasing concentrations of stearic or palmitic acid in the presence or absence of uncoupler as shown in fig.1. Non-esterified fatty acids did not significantly affect the activity and respiratory control of the enzyme. Only with stearic acid could a slight decrease in respiratory control index be read from the data. In contrast, a

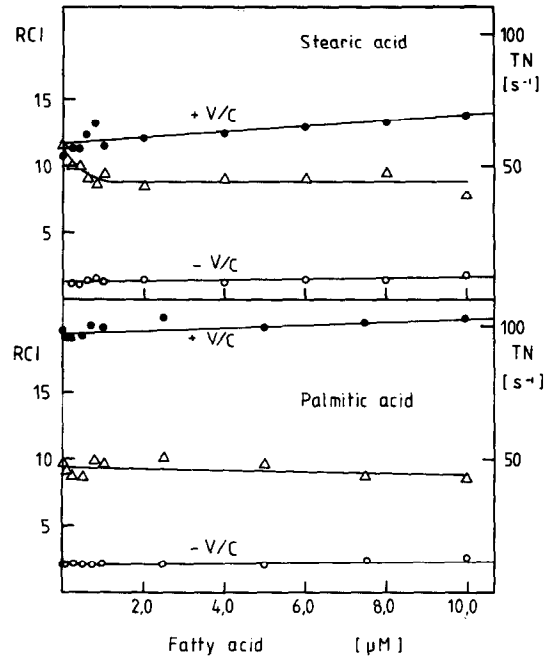

Fig.1. Effect of stearate and palmitate on oxygen uptake and respiratory control of reconstituted COX from bovine heart. Cholate-COX was reconstituted with asolectin. Respiration was measured in the presence (+V/C) and absence (−V/C) of uncoupler. Each value represents the average of two independent polarographic assays. RCI, respiratory control index.

clear decrease in respiratory control ratio was measured with the spectrophotometric assay (fig.2). This decrease resulted from a slight increase in the controlled and a decrease in the uncoupled rate of ferrocytochrome *c* oxidation. The maximal effect was obtained at 1 μM stearic or palmitic acid, no further decrease of respiratory control being found up to 10 μM fatty acid.

A more clearcut result was obtained when bovine heart COX was reconstituted in pure phosphatidylcholine containing 2% cardiolipin instead of reconstitution in asolectin (fig.3). Under these conditions, the decrease in respiratory control index amounts to 50% at 1 μM palmitic acid and remains unchanged up to 20 μM non-esterified fatty acid.

Attempts to isolate COX from bovine liver mitochondria, using cholate/deoxycholate as solubilizing reagent, were unsuccessful in obtaining respiratory control indices above 2–3, after reconstitution in asolectin liposomes. Therefore, COX was isolated from bovine heart and liver

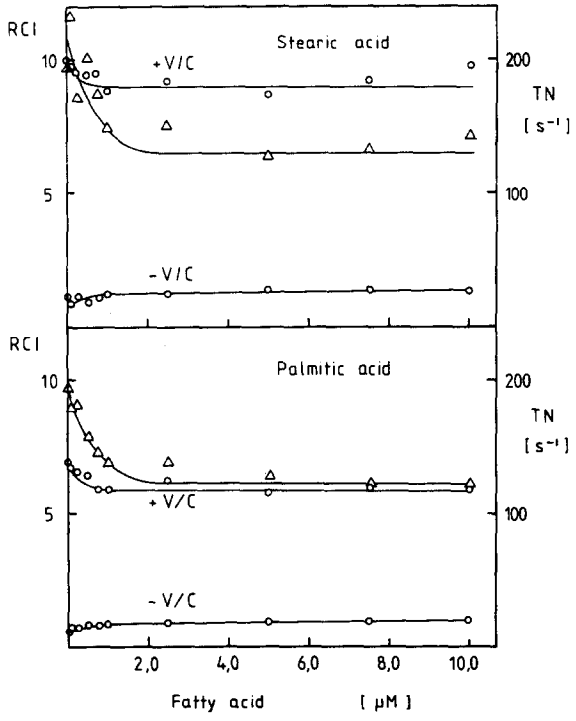

Fig.2. Effect of stearate and palmitate on the rate of ferrocytochrome *c* oxidation of reconstituted COX from bovine heart in the presence (+V/C) or absence (−V/C) of uncoupler and on the respiratory control index (RCI). Cholate-COX was reconstituted with asolectin. Each value represents the average of two independent spectrophotometric assays.

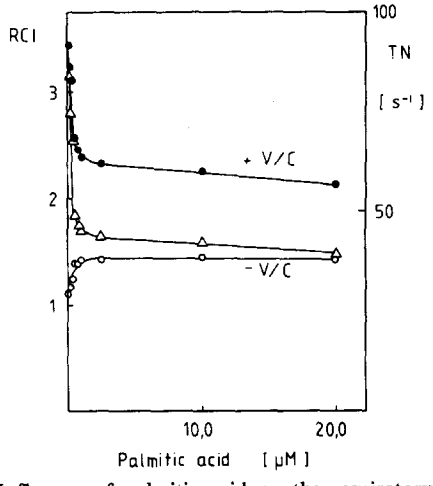

Fig.3. Influence of palmitic acid on the respiratory control index and ferrocytochrome *c* oxidation of bovine heart COX reconstituted with 98% phosphatidylcholine/2% cardiolipin. COX was isolated by the cholate method. Enzyme activity was measured in duplicate by the spectrophotometric method in the absence (−V/C) and presence (+V/C) of uncoupler.

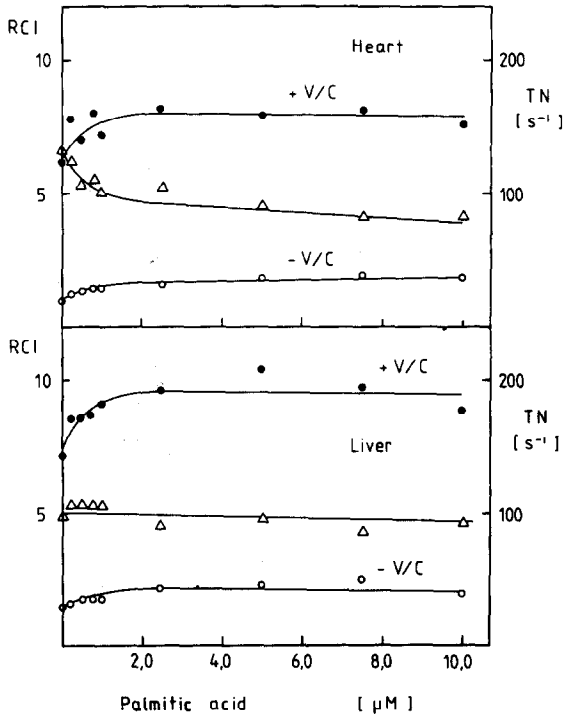

Fig.4. Different effect of palmitic acid on the respiratory control index of reconstituted COX from bovine heart and liver. The enzymes were isolated by use of Triton X-100 and reconstituted with asolectin. The spectrophotometric assay was performed with (+V/C) or without (−V/C) uncoupler. Each point is the average of two independent spectrophotometric assays.

mitochondria by using Triton X-114/Triton X-100 [10]. After reconstitution in asolectin liposomes these preparations had respiratory control indices of 5–7. As shown in fig.4, palmitic acid again influenced the rate of ferrocytochrome *c* oxidation within the concentration range up to 1 μM. In contrast to cholate-COX, however, the activity was stimulated in the presence as well as absence of uncoupler. The respiratory control index, however, decreased with increasing concentrations of palmitic acid with bovine heart, but not with the bovine liver enzyme. The lack of inhibition of respiratory control by palmitic acid of the liver enzyme cannot be due to the presence of non-esterified fatty acids in the enzyme preparation, since the maximal effect was obtained at a molar fatty acid/COX ratio of 50, whereas the isolated enzyme does not contain more than 4 mol phospholipid per mol COX [14].

4. DISCUSSION

Non-esterified fatty acids in the concentration range below 1 μM clearly influence the rate of ferrocytochrome *c* oxidation of reconstituted bovine heart COX, resulting in a 40–50% decrease in respiratory control ratio. The extent of this decrease apparently depends on the lipid composition of the liposomes. Above 1 μM fatty acids, no further influence was evident, as described by Labonia et al. [6].

Only negligible effects were observed when the respiratory activity of reconstituted COX was measured by the polarographic method. An effect of intraliposomal adenine nucleotides on the kinetics of ferrocytochrome *c* oxidation of reconstituted COX was also detectable only in the case of the spectrophotometric procedure and not with the polarographic method [15]. Because no effects of nucleotides were observed with COX from *Paracoccus denitrificans*, which lacks the 10 nucleus-encoded subunits of mammalian COX [16], we concluded that ATP and ADP interact with nucleus-encoded subunits of the complex [17]. The spectrophotometric assay represents the more 'physiological' method, because ferricytochrome *c* must dissociate from COX before the next ferrocytochrome *c* molecule can bind and transfer its electron. In contrast, the polarographic assay is assumed to involve re-reduction of enzyme-bound ferricytochrome *c* by ascorbate/*N,N,N',N'*-tetraphenyl-*p*-phenylenediamine [18,19]. It may thus be concluded that fatty acids, like intraliposomal nucleotides, influence the rate of ferricytochrome *c* dissociation from COX.

The decrease in respiratory control ratio of COX by non-esterified fatty acids may have physiological significance as it occurs within the concentration range of the K_m for non-esterified fatty acids of the cytosolic fatty acid binding protein [20], and would result in elevated nonshivering thermogenesis.

The opposite effect of non-esterified fatty acids on the uncoupler-stimulated activity was observed with cholate-COX and Triton-COX. The difference could be due to the presence of either a charged (cholate-COX) or an uncharged detergent molecule (Triton-COX) at the binding site for fatty acids. Charged lipids and detergents were found to affect strongly the activity of COX in a biphasic manner (Kadenbach, B. and Stroh, A., unpublished).

The data in fig.4 indicate a different response of COX from bovine heart and liver to non-esterified fatty acids. The two enzymes differ in 3 nucleus-encoded subunits [16,21]. The result could indicate a different involvement of the heart and liver in nonshivering thermogenesis, as postulated by Puchalski et al. [5]. It should be borne in mind, however, that different mechanisms may be involved in different tissues for nonshivering thermogenesis. Thus, a bypass of electron transport from extramitochondrial NADH was found in isolated rat liver mitochondria after cold exposure of rats [22]. Further work is necessary to characterize the interaction of non-esterified fatty acids with COX from liver mitochondria.

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