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Succinimidyl oleate, established inhibitor of CD36/FAT translocase inhibits complex III of mitochondrial respiratory chain

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ABSTRACT

The functional role of CD36 protein detected in mitochondrial fractions in long chain fatty acid (LCFA) oxidation is unclear due to conflicting results obtained in *Cd36* knockout mice and experiments using sulfo-*N*-succinimidyl oleate (SSO) for inhibition of CD36 mediated LCFA transport. We investigated effect of SSO on mitochondrial respiration and found that SSO substantially inhibits not only LCFA oxidation, but also oxidation of flavoprotein- and NADH-dependent substrates and generation of mitochondrial membrane potential. Experiments in rat liver, heart and kidney mitochondria demonstrated a direct effect on mitochondrial respiratory chain with the most pronounced inhibition of the complex III (IC_{50} 4 μM SSO). The results presented here show that SSO is a potent and irreversible inhibitor of mitochondrial respiratory chain.

Introduction

CD36/FAT (referred as CD36 hereafter) is an 88 kDa integral membrane glycoprotein and established component of the plasma membrane involved in long chain fatty acid (LCFA) transport [1]. In 1991 Harmon et al. found that sulfo-*N*-succinimidyl oleate (SSO) [2,3] binds to CD36 in plasma membrane and efficiently inhibits CD36-facilitated transport of LCFA. Since then SSO became widely used as specific inhibitor of CD36 in number of studies. Recently it has been proposed that CD36 also functions in the skeletal muscle and cardiac mitochondria [4]. Based on immunodetection of CD36 in isolated mitochondria as well as on effects of SSO [2,3] on mitochondrial oxidation of LCFA, it was proposed that CD36 is involved in LCFA transport to mitochondria, in concert with carnitine palmitoyltransferase I; however, the mechanism and potential interactions of CD36 with other mitochondrial components remain unclear (for review see [5]). CD36 protein was found in the outer mitochondrial membrane by mass spectrometry proteomic analysis [6] while no CD36 could be associated with mouse and human mitochondria by MitoCarta approach to mitochondrial proteome combining mass spectrometry, GFP tagging, and machine learning and identifying 1098 genes and their protein expression across 14 mouse tissues [7]. In 2007, the concept of mitochondrial CD36-dependent LCFA transport and oxidation was questioned by experiments with *Cd36* knock out mice [8]. As expected, no CD36 could be detected in plasma membrane or in mitochondria isolated from the $Cd36^{-/-}$ animals. However, there was no difference in the rate of LCFA oxidation by isolated mitochondria from muscle or heart of $Cd36^{-/-}$ animals as compared with control $Cd36^{+/+}$ mice, even when the oxidation of LCFA-CoA, as well as LCFA-carnitine esters in coupled or uncoupled state were analyzed in detail. Moreover, SSO was found to inhibit LCFA oxidation in both $Cd36^{-/-}$ and control animals. Recently, the same $Cd36^{-/-}$ animals [9] were reinvestigated by Holloway et al. [10] who detected a slightly lower LCFA oxidation and no exercise-dependent stimulation of LCFA oxidation in muscle mitochondria from knockout animals, while they observed that SSO had the same inhibitory effect on LCFA oxidation in both control and $Cd36^{-/-}$ mice. Moreover, they noted that SSO had also inhibitory effect on mitochondrial succinate respiration [10] which opens the question of the specificity of this inhibitor.

In the present work we therefore analyzed the effects of SSO on oxidation of different substrates by isolated mitochondria, electron transport activity of respiratory chain complexes and mitochondrial membrane potential. We have found that SSO effectively inhibits mitochondrial respiratory chain at several sites with most pronounced effect on complex III.

Materials and methods

Materials. All chemicals were of the highest commercially available purity from Sigma (Sigma–Aldrich Co., Germany). Sulfo-

Abbreviations: SSO, sulfo-*N*-succinimidyl oleate; LCFA, long chain fatty acids; TMPD, *N,N,N′,N′*-tetramethy1-*p*-phenylenediamine.

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N-succinimidyl oleate (SSO) was a kind gift of Prof. J.F.C. Glatz (Dept. Molecular Genetics, CARIM, Maastricht University). Stock solution of SSO was prepared in DMSO before each experiment.

Animals. We used adult Wistar-Kyoto (WKY/Ola) rats with a body weight of 180–220 g that were kept at a 12 h/12 h light/dark period with free access to standard laboratory chow and water. Animals were killed by decapitation in Narcotan narcosis. The experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), and were approved by the Ethics Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Isolated mitochondria. Isolation of mitochondria from liver, heart ventricle and kidney was carried out on ice or at 4 °C. Minced tissues were homogenized (10% homogenate, w/v, glass-Teflon homogenizer) in STE medium (0.32 M sucrose, 10 mM Tris–HCl, 1 mM EDTA, pH 7.4) and filtered through 200 μm nylon mesh before use. Liver and kidney homogenate was centrifuged 4 min at 1000 *g* and supernatant was centrifuged 2 min at 13,000*g*. Sedimented mitochondria were washed twice (2 min at 13,000*g*) and resuspended in STE medium. Heart homogenate was centrifuged 10 min at 600*g* and the supernatant was centrifuged for 5 min at 14,400*g*. Sedimented mitochondria were washed twice (5 min at 14,400*g*) and resuspended in STE medium. Mitochondrial proteins were determined by the Bradford method [11] using BSA as standard.

Respiration, membrane potential and spectrophotometric measurements. Oxygen consumption by isolated mitochondria was determined at 30 °C in K-medium (100 mM KCl, 10 mM Tris–HCl, 4 mM K-phosphate, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) as described before [12] using an Oxygraph-2k (Oroboros, Austria). The rates of oxygen consumption were normalized on protein content of mitochondria used and expressed as pmol s^{-1} mg $protein^{-1}$. Changes in mitochondrial membrane potential $\Delta\Psi_m$ of isolated mitochondria were analyzed at laboratory temperature in the K-medium using a TPP^+-selective electrode as described in [13]. The membrane potential was plotted as pTPP, *i.e.* natural logarithm of TPP^+ concentration (μM). Activities of mitochondrial enzymes, NADH:coenzyme Q reductase (complex I), succinate:DCPIP reductase (complex II), coenzyme Q:cytochrome *c* reductase (complex III) and cytochrome *c* oxidase (complex IV) in isolated mitochondria were measured spectrophotometrically by standard methods [14,15] at 30 °C.

Results

Inhibitory effect of SSO on mitochondrial respiration

Inhibition of LCFA oxidation by SSO at 50–200 μM [8,16,17] has been repeatedly used as a proof of the functional role of CD36 in mitochondria. Inhibitory effects of SSO on LCFA oxidation in mitochondria was considered specific due to the absence of any effect of SSO on pyruvate oxidation [16].

We have tested the effect of SSO on mitochondrial respiration using isolated rat liver mitochondria and different NADH- or flavoprotein-dependent substrates (Fig. 1). We have found an almost 80% inhibition of palmitoylcarnitine oxidation at 10 μM SSO but also a nearly complete inhibition of oxidation of succinate, pyruvate, glutamate and TMPD + ascorbate at 70 μM SSO. The inhibition of succinate or glutamate respiration showed a nearly linear relationship with the increasing SSO concentration, while the inhibitory curve of pyruvate oxidation was sigmoidal. The least sensitive was TMPD + ascorbate oxidation indicating a low sensitivity of cytochrome *c* oxidase. SSO thus exhibited rather complex effects on mitochondrial respiration and, in addition to inhibition of palmitoylcarnitine oxidation, affected directly components of the mitochondrial respiratory chain. SSO apparently binds to and interacts with several sites of the respiratory chain with a different sensitivity to this compound.

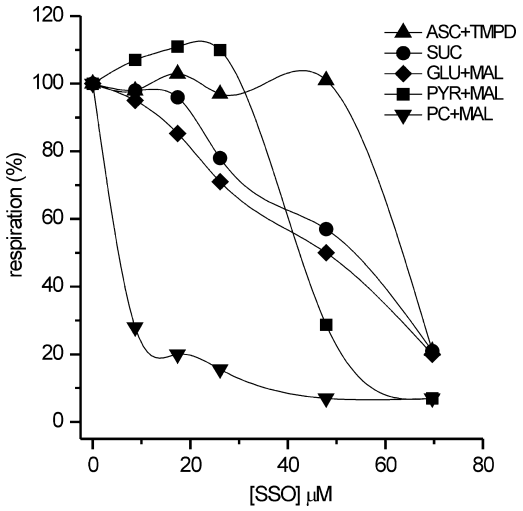

Fig. 1. Inhibitory effect of SSO on mitochondrial respiration with different substrates. ADP (1.5 mM)-stimulated respiration of isolated rat liver mitochondria was measured with 3 mM malate (MAL), 12.5 μM palmitoylcarnitine (PC), 10 mM succinate (SUC), 10 mM pyruvate (PYR), 10 mM glutamate (GLU), 5 mM ascorbate (ASC), 1 mM TMPD at indicated concentrations of SSO.

Effect of SSO on mitochondrial membrane potential $\Delta\Psi_m$

To test the effect of SSO on respiratory chain-induced energization of rat liver mitochondria, we measured the mitochondrial membrane potential using a TPP^+-selective electrode that allows for direct and continuous monitoring of changes in mitochondrial $\Delta\Psi_m$. As shown in Fig. 2A, when using succinate as substrate, high levels of $\Delta\Psi_m$ were observed at state 4, in the absence of ADP. The addition of SSO at 22 μM had a pronounced inhibitory effect and resulted in a complete collapse of mitochondrial $\Delta\Psi_m$ at 66 μM. A similar inhibitory effect of SSO on mitochondrial $\Delta\Psi_m$ was found when using both NADH- and flavoprotein-dependent substrates (glutamate + malate + succinate, Fig. 2B), although SSO was somewhat more inhibitory for succinate-induced $\Delta\Psi_m$.

Effect of SSO on individual components of respiratory chain

The inhibitory effect of SSO on respiration with succinate as well as with NADH-dependent substrates contrasts with a much lower sensitivity of cytochrome *c* oxidase and suggests that one of the SSO-inhibitory sites is apparently located upstream of complex IV and downstream of complex I. Therefore, we analyzed electron transport activities of individual respiratory chain complexes I, II, III and IV in the presence and absence of SSO using isolated rat liver mitochondria (Table 1). In accordance with respiration measurements, complex IV was much less inhibited than other respiratory chain complexes of which the complex III was the most sensitive. When using 200 μM SSO, complex IV retained 68% of its original activity, while complex III was completely inhibited. At 50 μM SSO, which is the lowest inhibitor concentration used for blocking CD36 in previous studies, the complex III was 98% inhibited and the inhibition of complexes I, II and IV accounted for 70%, 50% and 10% of the original activity, respectively. Detailed titration of the SSO-induced inhibition of complex III (Fig. 3) revealed IC_{50} of 4 μM SSO, demonstrating that SSO is also an efficient and relatively specific inhibitor of bc_1 complex of the mitochondrial respiratory chain. The same high sensitivity of complex III was found also in other types of mitochondria isolated from rat tissues – heart and kidney mitochondria (Fig. 3).

Succinimidyl forms stable adducts with primary amines ($R–NH_2$) located on peptides while the reactivity with aromatic amines, alcohols, and phenols, including tyrosine and histidine is