Activating ω-6 Polyunsaturated Fatty Acids and Inhibitory Purine Nucleotides are High Affinity Ligands for Novel Mitochondrial Uncoupling Proteins UCP2 and UCP3.*

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running title: ω-6 Polyunsaturated fatty acids and purine nucleotides as UCP2 and UCP3 ligands.

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SUMMARY

Human UCP2 and UCP3, expressed in yeast, were studied to establish their high affinity regulatory ligands. UCPn were reconstituted into liposomes and assayed for fatty acid-(FA)-induced H+ efflux. All natural long chain FAs activated UCP2- and UCP3-mediated H+ translocation. Coenzyme Q_{10} had no further significant activating effect. Evaluated parameters of FA activation (FA cycling) kinetics revealed the highest apparent affinity to UCP2 (the lowest K_m\,\text{s}, \,20 \,\mu M, \,29 \,\mu M, respectively) for \omega-6 polyunsaturated FAs (PUFAs), all-cis-8,11,14-eicosatrienoic and all-cis-6,9,12-octadecatrienoic acids, which are also the most potent agonists of the nuclear PPARβ receptor in the activation of UCP2 transcription. \omega-3 PUFA, cis-5,8,11,14,17-eicosapentaenoic acid had lower affinity (K_m 50 \,\mu M). Although being \omega-6 PUFA, arachidonic acid exhibited the same low affinity as lauric acid (K_m \sim 200 \,\mu M). These findings suggest a possible dual role of some PUFAs in activating both, UCPn expression and uncoupling activity.

UCP2- (UCP3)-dependent H+ translocation activated by all tested FAs was inhibited by purine nucleotides with apparent affinity to UCP2 (reciprocal K_i) decreasing in order: ADP > ATP ~ GTP > GDP >> AMP. Also ^3H-GTP (^3H-ATP) binding to isolated E.coli- (K_d \sim 5 \,\mu M) or yeast-expressed UCP2 (K_d \sim 1.5 \,\mu M) or UCP3 exhibited high affinity, similar to UCP1. The estimated number of ^3H-GTP high affinity (K_d <0.4 \,\mu M) binding sites was (in pmols/mg protein) 182 in lung mitochondria; 74 in kidney; 28 in skeletal muscle; and \sim 20 in liver mitochondria. We conclude that purine nucleotides must be the physiological inhibitors of the UCPn-mediated uncoupling \textit{in vivo}. 
INTRODUCTION

Novel mitochondrial uncoupling proteins, ubiquitous UCP2, predominantly muscle-specific UCP3, and brain-specific UCP4, and BMCP (or UCP5) form a gene subfamily within the mitochondrial anion carrier gene family together with "classic," brown-adipose-tissue-specific, UCP1, and at least three distinct plant UCPs (1,2). Three basic physiological roles of all UCPs should be manifested as consequences of low uncoupling states (2): i) a slight acceleration of metabolism due to slightly increased respiration; ii) a concomitant reduction in reactive oxygen species (ROS) formation (3, 4); and iii) inevitably related, mild thermogenesis. In addition, UCP2 (UCP3) most probably possesses more specific physiological roles (2, 5-14) in defense against excessive ROS production (2- 5), in participation in body weight regulation (5-8, 13); in heat production during various types of adaptive thermogenesis (8, 9,14), including fever (9), and in switching between pro- and anti-apoptotic processes (15). Pathologically distorted regulations of UCP2 (UCP3) expression or distorted ligand regulations are most probably among the causes of the development of obesity (13), type 2 diabetes (16), heart failure (5), and aging.

The diverse UCPn functions require fine-tuned regulations that cannot be accomplished only by regulation and upregulation of their transcription (e.g. 5,8,9,14,17,18) and by downregulation of their translation (19). Biochemical regulations, i.e. regulations by ligands, cofactors or by covalent modifications are also likely involved. The first regulation line could be provided by FAs (20). It has been established that, like UCP1 and plant UCP (PUMP), UCP2 and UCP3 are also activated by FAs (20, 21), most probably due to the FA cycling mechanism (20, 22-24). Hence, the availability of free FAs is the first prerequisite for UCP function. Second, purine nucleotides (PN) were confirmed to inhibit the protonophoric action of reconstituted E. coli-expressed UCP2 or UCP3. Garlid and coworkers observed a lower PN affinity toward UCP2 and UCP3 in sulfate media (20), while Klingenberg's group reported a higher affinity in low salt media (21). Modulation, i.e. the lowering of PN inhibition by some yet unknown factors may represent the mechanism which activates UCP2 or UCP3-mediated uncoupling in vivo. Such well-known modulators for UCP1 are alkaline pH and Mg$^{2+}$ (24, 25). Also, cofactors like Coenzyme Q$_{10}$ (21, 26) and superoxide (27)
were suggested to activate UCP2 (UCP3). Coenzyme Q_{10} was claimed to be required to restore the intact conformation of recombinant UCPs solubilized by sarcosinate from inclusion bodies (21, 26). Even native UCP2 (UCP3) was reported to be stimulated upon superoxide generation in various types of mitochondria (27). UCP2 (UCP3) in this presumably stimulated state was inhibited by ATP, ADP, GTP, and GDP in kidney or skeletal muscle mitochondria (27). These results suggested that native UCP2 (UCP3) could also be inhibited by PN. Recently, fluorescent nucleotide derivatives were demonstrated to interact with \textit{E. coli}-expressed UCP2 (28), but direct measurements with native nucleotides were still lacking.

Initial studies of recombinant UCP2 and UCP3 expressed in yeast were performed mostly while monitoring coupling in yeast mitochondria (6-8). Later, the available reports contain numerous contradictory results (29-36). Claims were made that UCP2 is specifically activated (state 4 respiration in yeast mitochondria is stimulated) by all-\textit{trans}-retinoic acid and that it is insensitive to palmitic acid, 9-\textit{cis}-retinoic acid and other FAs tested, including arachidonic, linoleic, docosahexaenoic acid, and prostaglandin E2 (36). It is difficult to imagine a physicochemical basis for such a strange FA specificity, incompatible with previous surveys of FAs interacting with UCP1 (37, 38).

Hence, in this work we have studied reconstituted yeast-expressed UCP2 and UCP3 in their partially purified (but highly active) form, in order to screen in detail their natural ligands, FAs and PN. We have clearly demonstrated that all natural long chain FAs, saturated or unsaturated, activate UCP2- or UCP3-mediated H^{+} translocation. From kinetics of putative FA cycling we have found the highest apparent affinity to UCP2 (lowest K_{m}) for two \omega-6 polyunsaturated FAs, which are also the most potent agonists of PPAR\textit{\beta} (17). This finding suggests their possible dual role in activating both UCP expression and uncoupling activity. We have also shown that the apparent affinity to UCP2 (reciprocal K_{i}) is decreasing in order: ADP > ATP ~ GTP > GDP >> AMP.

Demonstrating the existence of high affinity \textsuperscript{3}H-GTP and \textsuperscript{3}H-ATP binding to recombinant UCP2 (K_{d} \sim 1.5 \mu M for yeast expression) and UCP3, expressed either in yeast or \textit{E.coli} (K_{d}s \sim 5 \mu M) and showing that numbers of very high affinity \textsuperscript{3}H-GTP binding sites (K_{d}s \sim 0.2 - 0.4 \mu M) decrease in the mitochondria of lung > kidney > muscle > liver, we provide strong support for the view that PN must be the physiological inhibitors of the UCPn-mediated uncoupling \textit{in vivo}. 
EXPERIMENTAL PROCEDURES

Most of the chemicals were purchased from Sigma. \(^3\)H-labeled nucleotides were from Amersham Pharmacia Biotech. Hydroxylapatite, Bio-Gel HTP and Bio-Beads SM2 were from Bio-Rad. Octylpentaoxyethylene (OctylPOE) was from Bachem Feinchemikalien, Bubendorf, Switzerland. Materials for reconstitution were from the same sources as described elsewhere (39), materials for yeast fermentation were from Difco. Zymolyase 100T was from ICN. All other chemicals were of a reagent grade.

Yeast-expression of UCP2, UCP3

W303 yeast containing pCGS110 (or pYES) vectors with inserted cDNAs coding for human UCP2 and human UCP3 under control of an inducible Gal promotor and ura\(^\text{-}\) selection were donated by Ruth E. Gimeno and Louis A. Tartaglia (Millennium Pharmaceuticals, Inc., Cambridge, MA; 6). cDNA coding for UCP1 in pCGS110 vector contained in JB516 yeast was from Prof. Karl Freeman (Mc Master University, Hamilton, Ontario, Canada; 40). Yeast cells were grown on ura\(^\text{-}\) selective plates and were inoculated into 4 to 8 Erlenmayer 250 ml flasks with a medium containing 203 mM (1.5 vol\%) lactate, 0.05% glucose, 0.17% yeast nitrogen base (Difco) 0.5% ammonium sulfate, 0.005% each of L-aminoacids (tryptophan, methionine, arginine, leucine, histidine) and adeninesulfate. After 24 hours, 0.2% galactose was added and cells were shaken for another 24-28 hours until the optical density of 1 was achieved. Mitochondria were prepared immediately after terminating fermentation using Zymolyase 100T (ICN) to cleave the cell wall (40). Protein extraction followed immediately. Alternations in different fermentation yields were compensated by taking the same amount of yeast mitochondrial protein, usually 30 mg.

E. coli-expression of UCP2, UCP3

Bacterial strains BL21 (Novagen) containing plasmids pET21a with inserted cDNA coding for human UCP2 (or UCP3) open reading frames between the N\(\text{de}1\) and N\(\text{ot}1\) sites of the vector (Novagen) were donated by Dr. R.E.Gimeno and Louis A. Tartaglia (Millennium Pharmaceuticals, Inc., Cambridge, MA, 41). The cells were grown at 30\(^{\circ}\)C to OD600=0.6 and then induced with 1 mM IPTG at 30\(^{\circ}\)C for 6 hours. Cells from a 700 ml culture were lysed in a French Press in 20 ml of
lysis buffer (10 mM Tris pH 7, 1 mM EDTA, 1 mM DTT); the lysate was centrifuged at 27,000 x g for 15 min; and the pellet was resuspended in 20 ml of the lysis buffer and centrifuged at 1000g for 3 min. 1 ml aliquots of the supernatant were centrifuged at 14,000g for 15 min. The resulting pelleted inclusion bodies were stored frozen at -70°C. When used, they were suspended (3 mg of protein) and washed two times in 10 mM Tris-Cl, 0.1 mM Tris-EDTA, pH 7.0. The washed pellet was solubilized in 0.75 ml of 5 mM TEA-TES, 30 mM TEA$_2$SO$_4$, 0.1 mM Tris-EDTA, pH 7.2, containing 1.67% sodium lauroylsarcosinate (SLS) and 1% octylpenta(oxyethylene. The resulted micellar solution was concentrated (thus partly depleted of SLS) and subsequently diluted (usually 1:1) in 20 mM Na-MES, pH 6. Protein content was estimated using the Amidoblack method (42).

**Isolation and reconstitution of yeast-expressed UCP2, UCP3**

Reconstitution with lipid protection was adopted from Klingenberg (43) to comply with 6-methoxy-N-(3-sulfo-propyl)quinolinium (SPQ) fluorescent monitoring of ion fluxes (20, 22, 23, 37, 39). It included OctylPOE extraction of yeast mitochondria (30 mg protein) under lipid protection, the isolation step on HTP, detergent removal on Bio-Beads SM2 overnight, and external probe washing on Sephadex G25-300. The total amount of added lipid was 40.7 mg (egg yolk lecithin, type XI-E, Sigma, 4% cardiolipin and 1.6% L-α-phosphatidic acid). Additional lipids in 1 ml of final suspension, up to 20 mg, could have originated from the mitochondria. The internal medium for liposomes contained 84.4 mM TEA$_2$SO$_4$, 28.8 mM TEA-TES with 9.2 mM TEA, pH 7.2, 0.6 mM TEA-EGTA. The protein content of liposomes was also estimated by the Amidoblack method (42). Usually, lipid-to-protein ratio of about 1000 or higher was obtained. The identity of UCP2 (UCP3) in HTP-pass-through was verified using peptide mapping assisted by MALDI-TOF mass spectroscopy after in-gel trypsin cleavage of the PAGE-separated acetone-precipitated samples.

**Fluorescent monitoring of H$^+$ fluxes in proteoliposomes.**

Valinomycin-induced H$^+$ fluxes in the presence of various FAs were monitored by the SPQ quenching method (20, 22, 23, 37, 39) with 2 mM SPQ internally. Ethanol solutions of FAs were added to 25 µl (~1.5 mg) of vesicles in 2 ml of external medium (84.4 mM K$_2$SO$_4$, 28.8 mM TEA-TES with 9.2 mM TEA, pH 7.2, and 0.6 mM TEA-EGTA) and H$^+$ efflux was initiated by 0.1 µM valinomycin. Fluorescence was monitored on a RF5301 PC fluorometer (Shimadzu, Japan), equipped with
polarization filters (Polaroid) in cross-orientation in order to decrease light scattering. Fluorescence was calibrated to [H\(^+\)] by adding KOH aliquots to proteoliposomes in the presence of 1 \(\mu M\) nigericin and H\(^+\) flux rates were calculated as previously described (39). The internal volume was calculated from SPQ volume distribution (20, 22, 23, 39) assuming a total lipid content of 60.7 mg in 1 ml (1.5 mg in the assay) and typically amounted to \(\sim 1.2 \, \mu l\) (mg lipid)

**Assay for \(^3\)H-nucleotide binding**

Klingenberg’s anion exchange method was used to determine \(^3\)H-nucleotide binding (44). Either, *E.coli*-expressed proteins were used, or the isolation of yeast-expressed UCP2 and UCP3 was modified while the HTP-wash fractions were also taken. The samples were further desalted and concentrated using Amicon microconcentrators (cut off \(M_r\) 10 000), to obtain a concentrated micellar protein solution in 10 mM Na-MES, 1.5 mM TEA\(_2\)SO\(_4\), pH 6.5. The optimum protein to nucleotide ratio was adjusted to give linear Scatchard plots (45). Microliter aliquots of \(^3\)H-GTP (\(^3\)H-ATP) were added to a series of samples (50 \(\mu l\)) or to the parallel sample series with 2.5 mM GTP (ATP, respectively). After 60 min incubation at 20\(^\circ\)C, the unbound nucleotides were removed by the sample passage through 1 ml spin-columns containing Dowex, Cl\(^-\) form. The pass-through samples were subjected to liquid scintillation counting. The data were treated using Scatchard plot analysis (45).

**Assay for \(^3\)H-nucleotide binding in mitochondria**

Isolation of mitochondria from rat lung, kidney, skeletal muscle, and liver was performed essentially as described elsewhere (41). BSA-washed mitochondria (0.2 mg protein) were mixed with \(^3\)H-GTP aliquots (8-320 pmols; and with 0.6 mM “cold” GTP in parallel series) and incubated for 30 min in 200 \(\mu l\) of 100 mM sucrose, 20 mM Tris-HEPES, 1 mM EDTA, 2 \(\mu M\) rotenone, 5 \(\mu M\) CAT, pH 7.0. Mitochondria were then filtered through nitrocellulose filters (0.45 mm pores, Millipore). The filters were washed twice by the sucrose medium and placed into scintillation solution. The measured radioactivity of the samples containing an excess of non-radioactive GTP was always subtracted and Scatchard plots were constructed (45).
RESULTS

H⁺ efflux induced by fatty acids in proteoliposomes containing UCP2 and UCP3 - Figs. 1a,b illustrate the monitoring of H⁺ fluxes induced by K⁺ diffusion potential in the presence of lauric acid in proteoliposomes containing the recombinant uncoupling proteins, UCP2 or UCP3. Both were able to mediate H⁺ efflux upon the addition of 0.1 µM valinomycin to the vesicles pre-equilibrated with 200 µM lauric acid. The lauric acid addition causes the interior acidification of vesicles, called flip-flop acidification, reflecting the redistribution of FA molecules in both leaflets of the lipid bilayer (46). Note that the extent of this acidification is unchanged in the presence of ATP, reflecting the undistorted SPQ response. Consequently, we could detect that the observed H⁺ fluxes were up to 50% inhibited by various purine nucleotide (PN) di- and triphosphates (vide infra). High PN concentrations decreased also the extent of SPQ response to H⁺ efflux, which may reflect a possibility that a single protein per vesicle exists in our preparation (or if several proteins exist they have the same orientation). This is feasible due to our high lipid-to-protein ratio³ and such observations were made for years with UCP1 or plant UCP not only for SPQ monitoring of H⁺ fluxes, but also for Cl⁻ and alkylsulfonate fluxes (22,23,37,40,47).

Fig.1a,b illustrate the inhibition of the lauric acid-induced H⁺ efflux in proteoliposomes containing UCP2 or UCP3. However, ATP did not affect the slow H⁺ efflux induced at a 10-fold higher valinomycin concentration by lauric acid in proteoliposomes containing the extract from the mitochondria of W303 yeast not expressing UCP2 or UCP3 (Fig.1c). The W303-extract was prepared and passed via the HTP column in the same way as for yeast expressing UCP2 or UCP3. The resulting FA-induced H⁺ efflux increased with the increasing initial amount of W303 yeast mitochondria. It is likely mediated by yeast carriers of the HTP pass-through, such as the ADP/ATP carrier (48) or the phosphate carrier (39, 49). In turn, the ATP insensitivity of H⁺ efflux with the reconstituted W303-extract and only slight (<10%) inhibition of FA-induced H⁺ efflux in UCP2- or UCP3-proteoliposomes by CAT or methylenediphosphonate suggest that the ATP-sensitive H⁺ fluxes in UCP2- (UCP3)-proteoliposomes are indeed due to the UCP2 (UCP3) function, respectively.
Various natural FAs were able to induce H⁺ efflux in proteoliposomes containing UCP2 (Tab.I) and UCP3. Among all tested FAs, the fastest H⁺ efflux was found for oleic acid and myristic acids followed by polyunsaturated FAs. Also the H⁺ efflux induced by PUFAs was inhibited up to 50% by ATP present in the external medium (Fig.1d and vide infra). Similar results were found with UCP3. Moreover, additions of ω-6 PUFAs exhibited the highest extent of flip-flop acidification. Thus, cis-8,11,14-eicosatrienoic acid (C20:3 ω-6) had on average a double extent of flip-flop acidification when compared to lauric acid (Fig.1a,d). This finding would suggest that the physico-chemical properties of FA movement in the membrane and their partition coefficient Kp could contribute to their maximum cycling rates. Also flip-flop rate of fatty acids was previously found to increase with increasing unsaturation (50). In order to elucidate the involved structure/kinetic relationships we further studied the kinetics of the FA activation of UCP2-mediated H⁺ efflux.

**Kinetics of UCP2-mediated H⁺ efflux induced by lauric acid** - Kinetics of lauric acid activation of H⁺ efflux (FA cycling) in UCP2 proteoliposomes is illustrated in Fig. 2a. H⁺ efflux rates were evaluated with and without 2.5 mM ADP. Assuming Michaelis-Menten kinetics, we constructed Eadie-Hofstee plots for total rates (right panel in Fig. 2a, Table I) and for differential rates (not shown), when the rates measured with ADP were subtracted from the control rates. The kinetic parameters obtained for differential rates were used to construct the theoretical fit by the Michaelis-Menten equation (dotted line in Fig. 2a). This fit reflects the kinetics of uniformly oriented UCP2 molecules with the PN-binding site exposed outside. Mostly, this kinetics was similar to that measured with external ADP or ATP, which reflects the opposite UCP orientation.

The Eadie-Hofstee plot for the experiment with externally added ADP is nearly parallel to the control plot (Fig. 2a). Thus, kinetics in the absence and presence of ADP exhibits almost equal Km, while Vmax with ADP was about half of the control. As with UCP1, this fact reflects a noncompetitive and perhaps an allosteric type of nucleotide inhibition. Although subjected to errors, the differential rates also yielded an Eadie-Hofstee plot parallel with those for control or ADP. The apparent Kms for lauric acid and UCP2 or UCP3 (Tab. I) were higher than those found for UCP1 (22). Note, that this has been also observed for CoQ10-activated E.coli-expressed UCP2 and UCP3 (21).
Kinetics of UCP2-mediated H+ efflux induced by naturally abundant fatty acids - Among naturally abundant FAs, myristic acid, palmitic acid (Fig. 2b), oleic acid (Fig. 2c), linoleic acid, and arachidonic acid (Fig. 2d) exhibited the ability to induce H+ efflux in proteoliposomes containing UCP2 (Tab. I) or UCP3 (not shown). The saturated increase of the total H+ fluxes with the increasing total FAs was observed for all FAs tested. When comparing H+ flux densities per square µm (39) (Tab. I), the densities in UCP2- or UCP3-proteoliposomes were much higher than the densities for background H+ fluxes in the protein-free liposomes. They were usually also higher than the H+ flux densities estimated in the vesicles containing extracts from W303 yeast mitochondria (Tab. I). The Eadie-Hofstee plots constructed for total H+ fluxes in µmol H+·min⁻¹·(mg protein)⁻¹ were again parallel with those for H+ fluxes in the presence of external ATP or ADP and mostly also with those for differential rates (dotted lines in Fig. 2 a-d).

The apparent affinity for various FAs (taken as the inverse Kₘ, Tab. I) was quite similar but decreasing in order for heptylbenzoic > palmitic > lauric > linoleic > arachidonic > oleic > myristic acid. Note that PUFAs (vide infra) and nonphysiological heptylbenzoic acid exhibited higher affinity than naturally abundant FAs. The Vₘₐₓ values and turnover numbers per dimer are also listed in the Table I. The turnovers represent the minimum estimates, since not all measured protein is likely to be active. Among abundant FAs, the highest turnover was found for oleic acid, while it decreased for myristic > linoleic > arachidonic ≥ palmitic ≥ lauric acid. Heptylbenzoic acid exhibited the lowest evaluated turnover. Only 10% H+ flux was observed for caprylic acid and less than 5% for 12-hydroxylauric acid, an inactive FA which is unable to flip-flop across the lipid bilayer (46). Note that H+ fluxes in the absence of FAs amounted to 7 to 10% of Vₘₐₓ for lauric acid (Tab. I). Similar results were found with UCP3 - the apparent Kₘₙₜₜₖₘ were 170 µM, 260 µM and 197 µM for lauric, myristic and oleic acid, respectively (Vₘₐₓ were 147, 286 and 343.10⁻⁶ pmol H+·µm⁻²).

Do exceptionally activating fatty acids exist for UCP2? - Among the naturally abundant FAs tested as the above initial series, we have found almost no "exceptional" FAs that would induce significantly higher H+ fluxes in UCP2-proteoliposomes or a high apparent affinity, given by very low Kₘ. Table I indicates that oleic and myristic acid exhibited the highest rates, but also quite high Kₘ, reflecting the low affinity. Unsaturation was not a major factor, since arachidonic acid (C20:4 ω-6 Polyunsaturated fatty acids and purine nucleotides as UCP2 and UCP3 ligands
ω-6 Polyunsaturated fatty acids and purine nucleotides as UCP2 and UCP3 ligands

6) behaved quite similarly to palmitic and lauric acid. Nevertheless, we found more potent FAs when the kinetics of PUFAs was tested. Thus, cis-8,11,14-eicosatrienoic (or di-homo-γ-linolenic, C20:3 ω-6) and cis-6,9,12 octadecatrienoic (γ-linolenic, C18:3 ω-6) were found to induce very fast H^+ efflux in UCP2-proteoliposomes. However, their kinetics exhibited the lowest K_m found, 20 and 29 µM, respectively (Fig. 3, Tab. I). Similar results were found for UCP3. Also, cis-5,8,11,14,17-eicosapentaenoic (EPA, C20:5 ω-3, Tab. I) and cis-4,7,10,13,16,19 docosahexaenoic acid (C22:3 ω-3) were more efficient than FAs of the basic series of naturally abundant FAs. EPA exhibited the third highest apparent affinity (Tab. I).

**No effect of Coenzyme Q_{10} on UCP2 and UCP3-mediated H^+efflux induced by lauric acid** -
We have also tested the effect of the presumed activating cofactor, Coenzyme Q_{10}. No effect of oxidized CoQ_{10} (1 to 5 µM) was observed when added directly to the assay (Fig.1a, right panel), nor when oxidized CoQ_{10} was added to lipids during extraction of yeast mitochondria and formation of vesicles (not shown). This indicates that in our reconstituted system the recombinant yeast-expressed UCP2 and UCP3 are intact and do not need further activation other than by FAs. These results, however, do not entirely exclude the existence of CoQ_{10} activation of UCPs, since the effective CoQ_{10} dose could be extracted from yeast mitochondria. Nevertheless, with *E. coli*-expressed proteins CoQ_{10} activation did not exceed 30% - 1 µM CoQ_{10} at 100 µM lauric acid activated UCP2 to 132%; 0.1 µM CoQ_{10} activated UCP2 to 103%, UCP3 to 135%.

**Nucleotide inhibition of UCP2 and UCP3** - Also the above kinetic data demonstrated that the observed H^+ fluxes were up to 50% inhibited by various PN di- and triphosphates. The inability of complete inhibition by the external PN can be explained by two equally distributed orientations of UCP molecules in the membrane, with PN-binding sites exposed outside and inside (22). Hence, we could evaluate the inhibitory dose-responses for lauric acid-induced H^+ uniport while assuming a decrease to a 50% rate as 100% inhibition. Stock nucleotide solutions that are usually acidic had to be buffered by Tris-base and assay pH was carefully checked to be constant at 7.2, otherwise, artificial "inhibition" was observed due to a decrease in rates by acidic pH. For reconstituted UCP2, the lowest K_i was exhibited by ADP (350 µM, Fig. 4), while ATP exhibited higher K_i (445 µM) reflecting a slightly lower affinity of ATP to UCP2 (Fig. 4). These results were confirmed by three
independent experiments. The apparent affinity for GTP was slightly lower than for ATP (Tab. II) and for GDP it was even lower (Tab. II). The lowest affinity was found for AMP (Fig. 4, Tab. II); indeed it is similar to low affinity of AMP and GMP vs. ATP or GTP, previously documented for UCP1 (51, 52). Similar results were obtained with UCP3 (Tab. II). The magnitudes of $K_i$s for PN inhibition are higher than those found for UCP1, as also tested with recombinant yeast expressed UCP1 (Tab. II), but are lower than those reported for $E. coli$-expressed protein (20).

**Binding of $^3$H-GTP and $^3$H-ATP to isolated UCP2 and UCP3.** - Fig. 5 illustrates the typical Scatchard plots for $^3$H-GTP and $^3$H-ATP binding to yeast-expressed recombinant UCP2 preparation, while Tab. III summarizes results of several experiments. $^3$H-GTP binding in the presence of 1 $\mu$M CAT is also represented in Fig. 5, demonstrating binding under conditions eliminating possible contribution of the ADP/ATP carrier (CAT prevents nucleotide binding to this carrier and GTP has much lower affinity to it). The observed saturated binding was partially prevented by the unlabeled ("cold") GTP or ATP, respectively, but $^3$H-GTP binding was also prevented by ATP and ADP (not shown). Binding constants $K_d$s amounting in average $\sim$1.5 $\mu$M (Tab. III) were derived from the Scatchard plots for both, the GTP-sensitive portion of $^3$H-GTP binding, and the ATP-sensitive part of $^3$H-ATP binding. Taking into account the estimated protein content and assuming UCP2 as 100% pure, the calculated number of binding sites corresponded to 1.29 or 0.85 per UCP2 monomer without or with CAT, respectively (Fig. 5). $K_d$ for the experiment of Fig. 5 was 1.8 $\mu$M without CAT (a higher value could reflect contribution of the ADP/ATP carrier) and 1.68 $\mu$M with CAT. For $^3$H-ATP, $K_d$ changed from 1.5 $\mu$M (without CAT, Fig.5, Tab. III) to 1.3 $\mu$M with CAT, while the apparent number of binding sites decreased from 1.5 per UCP2 monomer by $\sim$10 to 20%.

Similar data but with lower affinities (higher $K_d$s) and lower numbers of binding sites were obtained for UCP2 and UCP3 expressed in $E. coli$ (Fig. 6; Tab. III). CAT had no effect (Fig. 6), thus reflecting that no ADP/ATP carrier contamination is possible with this expression (as well as no Coenzyme Q carryover). In spite of this, the obtained $K_d$s were three times higher, possibly also due to remaining lauroylsarcosinate present. We can conclude that the observed saturated binding reflects the nucleotide binding sites of recombinant UCP2 and UCP3 in both cases of expression.
The higher affinity and higher proportion of binding sites obtained for yeast-expressed proteins reflects the advantage of this system and the lipid protection used.

3H-GTP binding to isolated mitochondria of several tissues - In order to demonstrate the existence of native UCPs in intact tissues and to show the relevancy of the binding method not only for recombinant, but also for native proteins, we have evaluated 3H-GTP binding in the presence of CAT in mitochondria isolated from rat liver, skeletal muscle, kidney and lung (Fig. 7). In agreement with findings of Pequeur et al. (19), we found the highest number of 3H-GTP binding sites in lung mitochondria (182 ± 18 pmol/mg protein). This is about four times lower than the usual amount of 3H-GTP binding sites reflecting mostly the UCP1 molecules in BAT mitochondria (53). However, the evaluated Kd of 0.43 ± 0.03 μM reflects an even higher affinity than found with yeast-expressed human UCP2. The estimated total number of 3H-GTP binding sites was lower (74 ± 22 and 28 ± 6 pmol/mg protein, Kd's 0.3 ± 0.06 μM and 0.14 ± 0.02 μM) in kidney and skeletal muscle mitochondria, respectively, accounting for ~10 and ~30 times less than for UCP1 in BAT mitochondria. The lowest amount was found in liver mitochondria (21 ± 4 pmol/mg protein; Kd 0.23 ± 0.03 μM). The measured proportions between the numbers of 3H-GTP binding sites in the studied tissues correlate well to the proportions of UCP2 mRNA typically found in these tissues (5-7; 19). Hence, even if not all 3H-GTP binding sites could be ascribed to the native UCP2 (UCP2 and UCP3 in skeletal muscle), the interfering part should represent a minor portion. Consequently, these data represent the first demonstration of the existence of high affinity nucleotide binding to native UCP2 (UCP3).
DISCUSSION

In this work we have evaluated in detail the possible phenotypes of the novel uncoupling proteins UCP2 and UCP3. We have identified their best up-to-date known activating and inhibitory ligands. We have demonstrated that two (C18 and C20) \(\omega-6\) PUFAs are the most potent activators of UCP2 (UCP3), whereas among purine nucleotides, ADP is the most potent inhibitor. We have for the first time demonstrated the binding of natural \(\textsuperscript{3}H\)-labeled nucleotides to recombinant UCP2 (UCP3) proteins and to the mitochondria of several tissues.

Concerning activating FAs, we have clearly demonstrated that all physiologically abundant long chain FAs, saturated or unsaturated, activate \(H^+\) translocation in UCP2- and UCP3-proteoliposomes. This is demonstrated by the parameters of their activating (FA cycling) kinetics. We cannot explain why Rial \textit{et al.} (36) could not find any response with all their tested FAs except for all-\textit{trans}-retinoic acid in yeast mitochondria of yeast expressing UCP2. Among our tested FAs, we have found that oleic acid exhibited the highest rate, but \(\omega-6\) PUFAs, such as \textit{cis}-8,11,14-eicosatrienoic (C20:3 \(\omega-6\)) and \textit{cis}-6,9,12 octadecatrienoic (C18:3 \(\omega-6\)) exhibited both high \(V_{\text{max}}\) and the highest apparent affinity \((1/K_m)\). As such they were very efficient in inducing \(H^+\) uniport mediated by UCP2. Hence, we have shown that "more efficient" FAs do indeed exist and are activating UCP2-mediated \(H^+\) uniport in lower concentrations (amounts) than those required for other natural FAs. Their low \textbf{in vivo} abundance is balanced by their high activating profile. This is valid for the two \(\omega-6\) PUFAs which were identified by us as the best UCP2 activators. For example, the typical content as 1.9 and 2.9 \(\mu\)g of C20:3 \(\omega-6\), or 0.8 and 0.4 \(\mu\)g of C18:3 \(\omega-6\), was identified \textit{per} mg of phospholipids in rat liver and kidney, respectively (54). The amount of C20:3 \(\omega-6\) in human plasma phospholipids is equivalent to 100 \(\mu\)M (55). One can speculate that if 10% of these amounts would be cleaved off, a substantial activation of UCP2 will occur. A slightly lower efficiency with regard to UCP2 was found for \(\omega-3\) PUFAs, EPA (C20:5 \(\omega-3\)) and docosahexaenoic acid (C22:6 \(\omega-3\)), but due to the very high content of the latter in the brain or retina tissues (56), activation of UCP2 by C22:6 \(\omega-3\) is very plausible. The efficiency of C20:5 \(\omega-3\) and C22:6 \(\omega-3\) was still higher than that of arachidonic or oleic, or lauric and palmitic acid (equally effective as lauric). Our results have a great physiological relevance, since \(\omega-6\) PUFAs are among the most efficient activators of PPAR\(\beta\)
(17) and together with ω-3 PUFAs also to potent activators of PPARγ (18) and PPARα (57). In conclusion, our findings suggest their possible dual role in activating both UCP2 (UCP3) expression and the uncoupling activity.

Our results have also shown that protonophoric phenotypes of UCP2 and UCP3 do not differ qualitatively from the UCP1 phenotype. Their per-dimer-turnovers (estimated from V_{max} and total protein) fall into the range of hundreds per second (Table II). It is similar to the turnover of 94 s^{-1} that can be derived from the rate of 86.7 µmol H^{+} min^{-1}(mg protein)^{-1} for lauric acid at 25°C and CoQ_{10}-activated E. coli-expressed UCP2 (21). Also, the maximum per-dimer-turnover reported for UCP1 (133 s^{-1}, 26) is comparable. The same is true for plant UCP (99 s^{-1},47). The situation is more complex when one compares experimental apparent affinities taken as 1/K_{m}. Since, we did not subtract a contribution of non-protein related H^{+} leak, the real K_{m} values could be lower. Our K_{m} values (except for a few or those for PUFAs) are higher than those reported for UCP1 and for the phosphate carrier (49). Nevertheless, similar high K_{m} values can be derived from the results reported by Klingenberg (21).

Concerning PN inhibition, the derived K_{s} for UCP2 and UCP3 were higher than those for UCP1 in our reconstituted system, but all K_{s} for ATP and ADP were within the range of hundreds of µM. We must admit that sulfate used in our assay media strongly decreases the PN affinity to UCP1 (25) and presumably also to UCP2, UCP3, which might explain the discrepancies between our K_{s} and those measured by Klingenberg (21, 58) and partly also between the K_{s} (Tab. II) and binding constants K_{d}s (Tab. III). We chose sulfate to ensure that the anion used would be membrane impermeant, would not quench the SPQ probe, and that UCPs would not transport it. Thus, sacrificing the efficiency of nucleotide inhibition, we ensured high K^{+} diffusion potential and the correct probe response. Our media may better simulate in vivo conditions. We have also demonstrated that ADP is a slightly stronger UCP2 inhibitor than ATP. The same finding has previously been reported for UCP3 (58). GTP and GDP exhibited a little bit lower inhibitory ability. However, the differences between ATP and GTP were not found for binding constants K_{d}s. It is also not certain whether the observed small difference in ATP and ADP affinities in vitro can
account for the suggested *in vivo* activation of UCP3 by the increased ATP/ADP ratio (58), simply due to higher ADP affinity.

We have also confirmed the existence of PN interaction with UCP2 (UCP3) by the direct binding experiments using $^3$H-GTP and $^3$H-ATP. In spite of the fact that the binding of fluorescent nucleotide derivatives was reported recently (28), our results represent the first measurements with natural PNs (Figs. 5-7). We have evaluated equal binding constants $K_{ds}$ and nearly an equal number of binding sites for $^3$H-GTP, and $^3$H-ATP for yeast-expressed proteins. Also, the number of PN-binding sites was not reduced to zero with CAT and actually amounted roughly to 1 with CAT. This indicates that if binding to the ADP/ATP carrier contributes to the total binding in the samples obtained by yeast-expression, it represents a minor contribution. The data obtained with CAT then reflect net binding to UCP2. For *E. coli*-expressed UCP2 and UCP3, similar data were found, but with higher $K_{ds}$s and lower binding site numbers. This may reflect the continued presence of interfering lauroylsarcosinate or that only a portion of the population of UCPn molecules has an intact conformation. Nevertheless, the affinity derived as reciprocal $K_d$ is about three times higher for the native UCP2 in mitochondria than for recombinant yeast-expressed UCP2. Obviously, the natural insertion of UCP2 molecule in the internal membrane is superior to the micellar solution.

The existence of high affinity $^3$H-GTP-binding sites in mitochondria suggests that these sites may indeed reflect the binding to native UCP2 (UCP2 plus UCP3 in skeletal muscle mitochondria). This conclusion is strongly supported by our finding of the highest number of $^3$H-GTP binding sites in the mitochondria of tissues where UCP2 content was reported to be high, *i.e.* in lung (19) and kidney (27). On the contrary, we found a ~10 times lower number of $^3$H-GTP-binding sites in the mitochondria of liver, an organ which has almost no UCP2 expression (6,7), unless it is stimulated, *e.g.* by cytokines (2,9). This fact again suggests that the detected $^3$H-GTP-binding sites could be predominantly formed by UCP2. Surprisingly, skeletal muscle mitochondria, presumably containing both UCP2 and UCP3, exhibited only a little bit higher content of $^3$H-GTP-binding sites than liver mitochondria. However, this estimate fits well with the protein measurements using antibodies (19). In conclusion, the maximum UCP2 content found in lung mitochondria (~200 pmol/ mg protein, Fig. 7) is about four times less abundant than the content of UCP1 in BAT
mitochondria (53). In skeletal muscle, the UCP2 + UCP3 content derived from the total number of 
$^3$H-GTP-binding sites was ~7 times smaller than the UCP2 in the lung, and in the liver, UCP2 is 
even less abundant. The finding of small amounts of UCP2 in isolated mitochondria supports the 
concept of UCP2 (UCP3) as a stress protein, which can be elevated as required under various 
physiological situations (2, 5).

It would seem to be trivial to note that no superoxide activation was required in our experi-
ments to demonstrate PN-binding or FA-activated H$^+$ uniport. But, since other authors claim that 
only under such activation can PN-inhibition of FA-induced uncoupling be observed (27), we must 
stress this point. In conclusion, we have demonstrated that yeast-expressed UCP2 and UCP3 are 
able to mediate PN-sensitive H$^+$ transport in the presence of FAs and that recombinant yeast- or E.
coli- expressed UCP2 and UCP3 do indeed bind purine nucleotides. The latter property is reflected 
in vivo by the various number of PN-binding sites in the mitochondria of various tissues. Their 
number, and hence the amount of UCP2 (UCP3) is, however, quite low – except for lung 
mitochondria, it is at least 10 times lower than the amount of UCP1 in BAT mitochondria. In spite of 
this fact, in vivo, when not all FAs are metabolized or when FAs would be cleaved off lipids (e.g. by 
phospholipase C), a certain non-zero uncoupled state may exist in all types of mitochondria. Such 
a weak uncoupling state would be enabled by the lowering (modulating) PN affinity due to elevated 
concentrations of anions, Mg$^{2+}$, or other putative modulators of PN inhibition. The resulting weak 
uncoupling also prevents the excessive production of reactive oxygen species. Yet, the unknown 
regulatory mechanisms may further release PN inhibition to achieve a higher or complete 
activation of UCPn-mediated uncoupling. The states of higher uncoupling would then represent the 
specific thermogenic roles of UCPn in different tissues, such as tissue-specific adaptive 
thermogenesis, and simultaneously, a state with a very low mitochondrial production of the reactive 
oxygen species.
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**FOOTNOTES:**

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*ACKNOWLEDGEMENT:*

Excellent technical assistance of Jana Brucknerová and Jana Košařová is gratefully acknowledged as well as the help of Petr Hanák, M.S., with yeast expression, and of Tomáš Špaček, M.S., with some binding assays. The project was supported by the research project AVOZ5011922 and by the grants of the program Kontakt from the Czech Ministry of Education (ME 389), of Grant Agency of the Czech Republic, No. 301/02/1215, and by the Internal Grant Agency of the Academy of Sciences of the Czech Republic (No. A5011106). Fluorometer was purchased from the funds of the Czech-U.S. Science and Technology Program, grant No. 86043.

1 abbreviations: BAT - brown adipose tissue; CAT - carboxyatractyloside; EPA - *cis*-5,8,11,14,17-eicosapentaenoic (C20:5 ω-3) acid; FA - fatty acid; FCCP - carbonyl cyanide trifluoromethoxyphenylhydrazone; HTP - hydroxylapatite; MES - 2-[N-morpholino] ethanesulfonic acid; OctylPOE - octylpentaoxyethylene; PN - purine nucleotides; UCP1 - uncoupling protein of brown adipose tissue mitochondria; UCP2 - "ubiquitous" uncoupling protein; UCP3 - skeletal muscle-specific uncoupling protein; UCPn - any uncoupling protein; SPQ - 6-methoxy-N-(3-sulfopropyl)quinolinium; SLS - sodium lauroyl sarcosinate; TEA - tetraethyl ammonium; TES - N-tris [hydroxymethyl]methyl-2-amino-ethanesulfonic acid.;

2 unpublished data of Ježek *et al.* have shown inhibition of fatty acid cycling via ADP/ATP carrier by agaric acid.

3 at much lower lipid-to-protein ratio in much larger vesicles about 6 proteins per vesicle were estimated by Winkler, E., and Klingenberg, M., *Eur.J. Biochem.* 207, 135-145, 1992

4 unpublished data of Ježek *et al.*
LEGENDS TO FIGURES:

Fig. 1. ATP sensitivity of lauric acid-induced H⁺ fluxes in proteoliposomes containing UCP2 (a,d), UCP3 (b), or hydroxylapatite-passed extract from W303 yeast mitochondria (c)

Typical traces of intraliposomal H⁺ (monitored by SPQ) are shown for proteoliposomes equilibrated with 200 µM lauric acid ("LA", a-c) or 50 µM cis-8,11,14-eicosatrienoic acid (ETA, d). The FA addition leads to so-called flip-flop acidification, resulting from establishing equilibrium with regards to both membrane and acid-base-equilibrium (46). A subsequent addition of 0.1 µM valinomycin ("Val", 1 µM for c)) leads to internal alkalization indicating H⁺ efflux. This H⁺ efflux was inhibited up to 50% by ATP, but not for the reconstituted HTP-passed detergent extract of W303 yeast mitochondria containing the empty expression vector (c). The right panel in a) shows no effect of 1 µM Coenzyme Q10 (+CoQ10).

Flux densities in 10⁻⁶ pmol H⁺ s⁻¹ µm⁻² were estimated as follows: a) UCP2 - control: 62; 1.87 mM ATP: 34; 1 µM Coenzyme Q₁₀: 61 b) UCP3 - control: 78; 0.75 mM ATP: 40. c) control: 42; 1.87 mM ATP: 42. d) control: 108; 1.87 mM ATP: 48. Control rates expressed in µmol H⁺ min⁻¹(mg protein)⁻¹ were 68 for UCP2 (a) 146 for UCP2 with ETA (d), and 62 for UCP3 (b).

Fig. 2. Kinetics of presumed fatty acid cycling mediated by UCP2

a) lauric acid; b) palmitic acid; c) oleic acid; d) arachidonic acid.

Left panels show the direct plots of FA-induced H⁺ flux with increasing total FA concentration for the absence (filled symbols) and presence (open symbols) of 2.5 mM ATP (ADP for a), while the right panels illustrate Eadie-Hofstee plots for the same data. The dotted lines represent theoretical fits by the Michaelis Menten-equation for the differential H⁺ fluxes, when fluxes in the presence of nucleotides were subtracted from the control ones (Kᵣ and Vₘₐₓ were taken from the linearized Eadie-Hofstee plots). The obtained kinetics parameters are listed in Table I.
**Fig. 3.** Kinetics of $\omega$-6 eicosatrienoic acid-induced $H^+$ efflux in UCP2 proteoliposomes.

cis-8,11,14-Eicosatrienoic acid (ETA) -induced $H^+$ efflux initiated by 0.1 $\mu$M valinomycin in UCP2 proteoliposomes was measured for increasing ETA amounts in the absence (●) and presence of 2.5 mM ATP (○). The left panel contains the derived direct kinetic plots, while the right panel shows the corresponding Eadie-Hofstee plot, linearized without taking into account the two highest data points. At these high rates the total $H^+$ flux was influenced by a non-specific $H^+$ flux (as seen from the diminished ATP-inhibitory strength). The dotted lines represent theoretical fits by the Michaelis Menten-equation for the differential $H^+$ fluxes. The obtained $K_m$ and $V_{max}$ are listed in Table I.

**Fig. 4.** Nucleotide dose-responses for inhibition of lauric acid-induced $H^+$ fluxes in UCP2-proteoliposomes by: ADP (□), ATP (▼), and AMP (●)

Inhibition of $H^+$ fluxes induced by 200 $\mu$M lauric acid in proteoliposomes containing UCP2 was measured and the dose-responses were constructed on the assumption of maximum inhibition at a 50 % rate (main panel) from the data expressed in relative rates (inset). Solid lines represent fits by the Hill equation, which yielded the inhibitory constants $K_i$s 350 $\mu$M for ADP, 445 $\mu$M for ATP and 3140 $\mu$M for AMP.

**Fig. 5.** $^3$H- nucleotide binding to isolated yeast-expressed UCP2:

(□) $^3$H-GTP in the absence of CAT, (■)$^3$H-GTP in the presence of 1 $\mu$M CAT, (○)$^3$H-ATP in the absence of CAT

Yeast-expressed UCP2 was isolated and assayed with radio-labeled PN as described in the Experimental Procedures. The Scatchard plots are shown for two parallel measurements with subtracted data for background measured with 2.5 mM “cold” GTP or ATP, respectively. The derived binding constants $K_d$s were 1.8 $\mu$M for $^3$H-GTP without CAT; 1.68 $\mu$M for $^3$H-GTP with CAT; and 1.46 $\mu$M for $^3$H-ATP (single experiment). Assuming that preparation contains only a UCP2 protein (Mr~33 000), the calculated numbers of $^3$H-GTP binding sites corresponded to 1.29
per UCP2 monomer (1.5 for \(^3\)H-ATP) in the absence of CAT and to 0.85 per UCP2 monomer in the presence of CAT.

**Fig. 6.** \(^3\)H-GTP nucleotide binding to isolated *E. coli*-expressed UCP2 or UCP3: (◇) UCP2 in the absence of CAT; (O) UCP2 with 1 \(\mu\)M CAT; (▼) UCP3

*E. coli*-expressed UCP2 and UCP3 were isolated and assayed with radiolabeled PN as described in the Experimental Procedures. The Scatchard plots are shown with subtracted data for background measured with 2.5 mM “cold” GTP. The derived binding constants \(K_d\)s are 5.8 \(\mu\)M for UCP2 in the absence of CAT, 5.1 \(\mu\)M in the presence of 1 \(\mu\)M CAT (fit by the dotted line), and 7.27 \(\mu\)M for UCP3, while the calculated number of binding sites corresponded to 0.4 and 0.35 per UCP2 monomer in the absence or presence of 1 \(\mu\)M CAT, respectively, and to 0.35 per UCP3 monomer.

**Fig. 7.** \(^3\)H-GTP binding to isolated mitochondria from several rat tissues: (O) lung; (▲) kidney; (▼) skeletal muscle; (●) liver

The Scatchard plots are shown with subtracted data for background, measured with 2.5 mM “cold” GTP. Average values ± SDs are plotted for data measured on several mitochondrial isolations (n). The derived number of binding sites corresponded to 182 ± 18 pmol/mg protein, n = 4, in lung; to 74 ± 22 (n = 3), 28 ± 6 (n = 2), and 21 ± 4 pmol/mg protein (n = 5), in kidney, skeletal muscle, and liver mitochondria, respectively. ± Errors of x-intercepts were taken from linear regression fits. The derived binding constants \(K_d\)s were 0.43 ± 0.03 \(\mu\)M for lung; 0.3 ± 0.06 \(\mu\)M, 0.14 ± 0.02 \(\mu\)M, and 0.23 ± 0.03 \(\mu\)M in kidney, skeletal muscle, and liver mitochondria, respectively.
Table I. Kinetic parameters of putative fatty acid-cycling via mitochondrial UCP2

Kinetics, measured as H⁺ flux dependence on total fatty acid concentration, was evaluated from Eadie-Hofstee plots, such as on Fig. 2, and Fig. 3. (a at 100 µM)

| Fatty acid          | Relative rate at 200 µM (%) | $V_{\text{max}}$ ($10^{-6}$ pmol H⁺ s⁻¹ µm⁻²) | $V_{\text{max}}$ (µmol H⁺ min⁻¹ (mg prot)⁻¹) | max turnover per dimer (s⁻¹) | $K_{m}$ (µM) |
|---------------------|----------------------------|-----------------------------------------------|----------------------------------------------|-------------------------------|-------------|
| lauric acid         | 100%                       | 125                                           | 73                                           | 80                            | 185         |
| myristic acid       | 262%                       | 324                                           | 227                                          | 250                           | 450         |
| palmitic acid       | ~100%                      | 151                                           | 90                                           | 100                           | 178         |
| oleic acid          | 350%                       | 462                                           | 262                                          | 288                           | 334         |
| linoleic acid       | 170%                       | 136                                           | 172                                          | 189                           | 237         |
| arachidonic acid    | 118%                       | 180                                           | 103                                          | 114                           | 246         |
| C18:3 ω-6           | 325%<sup>a</sup>           | 220                                           | 190                                          | 209                           | 29          |
| C20:3 ω-6           | 200-300%<sup>a</sup>       | 204                                           | 205                                          | 226                           | 20          |
| C20:5 ω-3           | 220%<sup>a</sup>           | 272                                           | 235                                          | 258                           | 50          |
| heptylbenzoic       | 90%                        | 91                                            | 52                                           | 57                            | 130         |
| caprylic acid       | 11%                        | -                                             | -                                            | -                             | -           |
| 12-OH-lauric        | 5%                         | -                                             | -                                            | -                             | -           |
| UCP3 lauric         | -                          | 147                                           | 80                                           | 88                            | 170         |
| Protein:            | Fatty acid:                | Valinomycin-induced H⁺ efflux:                | Rate at 200 µM ($10^{-6}$ pmol H⁺ s⁻¹ µm⁻¹) |                  |
| UCP2                | lauric acid                | 80 ± 13 (n=23)                                | 67 to 86                                     |
| UCP3                | lauric acid                | 67 to 86                                     |                                               |
| UCP1                | lauric acid                | 95                                            |                                               |
| W303 extract        | lauric acid                | 43                                            |                                               |
| liposomes           | lauric acid                | 27                                            |                                               |
| liposomes           | palmitic acid              | 17                                            |                                               |
| liposomes           | oleic acid                 | 39                                            |                                               |
| Liposomes           | linoleic acid              | 19                                            |                                               |
| UCP2 proteoliposomes| no fatty acid              | 12 ± 3 (n=17)                                 |                                               |


**Table II. Inhibitory constants for nucleotide inhibition of UCPs**

Nucleotide inhibition of H^+ fluxes induced by lauric acid (200 µM, 100 µM in some cases) was measured for recombinant proteins expressed in yeast and inhibitory constants (K<sub>i</sub>s in µM) were evaluated on the assumption of maximum 50% inhibition by externally added nucleotides from fits by Hill equation. (n.d. not determined)

| Nucleotide | UCP2 | UCP3 | UCP1 |
|------------|------|------|------|
| ADP        | 350  | 173  | 330  |
| ATP        | 445  | 446  | 200  |
| GTP        | ~700 | 780  | n.d. |
| GDP        | 756  | 1000 | n.d. |
| AMP        | 3140 | n.d. | n.d. |

**Table III. Nucleotide binding constants for recombinant UCP2 and UCP3**

Table shows average values (± SDs) of nucleotide binding constants estimated from Scatchard plots (their number n, equal to the number of protein isolations, is listed in parentheses), measured as described in Experimental procedures.

| Nucleotide | Yeast-expressed | E. coli-expressed | Yeast-expressed | E. coli-expressed |
|------------|-----------------|-------------------|----------------|-------------------|
| ^3^H-GTP   | 1.4 ± 0.4 (n=10)| 5.6 ± 0.4 (n=12) | 2.5            | 5.7 ± 0.9 (n=8)   |
| ^3^H-ATP   | 1.5 ± 0.1 (n=3) | 5.1               | n.d.           | 5.6               |
Fig. 1b, c

b) LA
Val
+ATP

20 s

0.5 mM

20 s

0.5 mM

+ATP
Fig. 2b

![Graph showing the relationship between J (µmol H⁺ min⁻¹ mg protein⁻¹) and [palmitic acid] (µM). The graph includes a curve and data points. The right panel shows a linear relationship between J / [palmitic acid] and J (µmol H⁺ min⁻¹ mg protein⁻¹).]
Fig. 2c

Graph showing the relationship between $J$ (μmol H⁺ min⁻¹ (mg protein)⁻¹) and [oleic acid] (μM) on the left, and $J / [oleic acid]$ on the right.
Fig. 3

The figure shows two graphs. The left graph illustrates the relationship between [eicosatrienoic acid] (μM) and $J$ (μmol H⁺ min⁻¹ mg protein⁻¹). The right graph shows the relationship between $J / [eicosatrienoic acid]$ and $J$ (μmol H⁺ min⁻¹ mg protein⁻¹).
Fig. 4

[Graph showing inhibition and relative rate as a function of nucleotide concentration.]

Inhibition (%) vs. [nucleotide] (µM)
Fig. 5

$^{3}$H-GTP or $^{3}$H-ATP bound (pmol/mg protein)

Bound / free

0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7

0 10000 20000 30000 40000

$^{3}$H-GTP or $^{3}$H-ATP bound (pmol/mg protein)
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*J. Biol. Chem. published online April 1, 2003*

Access the most updated version of this article at doi: 10.1074/jbc.M212850200

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