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Perfluorooctane sulfonamide: a structurally novel uncoupler of oxidative phosphorylation

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The effects of sulfluramid (*N*-ethylperfluorooctane sulfonamide) and perfluorooctane sulfonamide (DESFA) on isolated rabbit renal cortical mitochondria (RCM) were examined. Sulfluramid (1–100 μM) and DESFA (0.5–50 μM) increased state 4 respiration of RCM respiring on pyruvate/malate or succinate in a concentration dependent manner in the absence of a phosphate acceptor. In addition, both sulfluramid and DESFA increased state 4 respiration in the presence of oligomycin, an inhibitor of F_0F_1-ATPase. The effects of sulfluramid (200 μM), DESFA (100 μM), and the known protonophore and uncoupler of oxidative phosphorylation, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 μM), on RCM proton movement were examined directly by monitoring extramitochondrial pH and indirectly by monitoring passive mitochondrial swelling. Immediately upon addition, DESFA and FCCP, but not sulfluramid, dissipated the RCM proton gradient and caused RCM to swell in solutions of NaCl or NH_4Cl. These results show that DESFA uncouples oxidative phosphorylation by acting as a protonophore. RCM were shown to metabolize sulfluramid to DESFA which suggests that the increase in state 4 respiration observed with sulfluramid is due to DESFA. DESFA is unique in that it is one of two uncouplers that does not contain a ring structure and thus may be a useful model in the study of oxidative phosphorylation.

Introduction

The halogenated alkyl sulfonamide, sulfluramid (*N*-ethylperfluorooctane sulfonamide) is currently being marketed as an insecticide for cockroaches and ants. In addition, the red imported fire ant (*Solenopsis invicta* Buren) has been shown to be very sensitive to sulfluramid [1,2]. Recently, Schnellmann [3] reported that sulfluramid and its metabolite, perfluorooctane sulfonamide (DESFA) were potent stimulators of renal proximal tubule basal oxygen consumption (Q_{O_2}), with initial effects exhibited at 5–10 μM and maximal effects at 50–200 μM. The increase in Q_{O_2} was ouabain-insensitive, suggesting that sulfluramid and/or DESFA may act by uncoupling oxidative phosphorylation. Furthermore, sulfluramid and DESFA increased state 4 respiration of isolated rabbit renal cortical mitochondria (RCM). Tubular death occurred following 60 min exposures to sulfluramid or DESFA at concentrations of 100 μM or greater. These results suggested that sulfluramid and/or DESFA uncouple oxidative phosphorylation and may produce cytotoxicity through this mechanism. The purpose of the study reported herein was to determine: (1) whether sulfluramid and/or DESFA uncouple oxidative phosphorylation, (2) the mechanism by which they produce their effects on RCM, and (3) whether RCM metabolizes sulfluramid to DESFA. If sulfluramid or DESFA uncoupled oxidative phosphorylation, it would join compound 1799 [4] as an additional representative of a non-ring-containing uncoupler and might be used as a model to study oxidative phosphorylation.

Experimental procedures

Materials. Sulfluramid and DESFA were obtained from Griffin (Valdosta, GA). [*N-ethyl*-1-^{14}C]Sulfluramid (14.1 mCi/mmol) was purchased from Dupont (Boston, MA). ADP, antimycin A, and rotenone were obtained from Sigma (St. Louis, MO). FCCP was

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Q_{O_2}, oxygen consumption; DESFA, perfluorooctane sulfonamide; RCM, renal cortical mitochondria.

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purchased from Aldrich (Milwaukee, WI). All other reagents were at least reagent grade quality.

Renal cortical mitochondria. Washed RCM were isolated from female New Zealand White rabbits (2–3 kg) (Myrtle's Rabbitry, Thompson Station, TN) by the method of Weinberg and Humes [5]. The isolation buffer consisted of 0.27 M sucrose, 5 mM Tris-HCl and 1 mM EGTA. The final mitochondrial pellet was resuspended in 0.27 M sucrose. The mean respiratory control ratio (state 3 respiration/state 4 respiration) [6] for RCM respiring on pyruvate/malate (5/5 mM) used in these studies was 6.4 ± 0.3 (mean ± S.E.).

Oxygen consumption (Q_{O_2}). The Q_{O_2} of mitochondrial suspensions was monitored using a Clark-type oxygen electrode and an oxymeter. The chamber (1.5 ml) was warmed to 37 °C and stirred magnetically. The incubation medium consisted of 130 mM KCl, 9 mM Tris-PO_4, 4 mM Tris-HCl, 1 mM EGTA (pH 7.3) and 1.3 mg of mitochondrial protein. Respiratory substrates were either potassium pyruvate (5 mM) + potassium malate (5 mM) or potassium succinate (10 mM). When potassium succinate was the respiratory substrate, 10 μM rotenone was present. For specific experiments see individual tables and figures.

Mitochondrial swelling. RCM swelling was determined using the method of Cunarro and Weiner [7,8]. Briefly, an aliquot of mitochondria (0.6 mg protein) was added to a continuously stirred cuvette containing 10 mM triethanolamine-HCl buffer, 5 mM potassium pyruvate, 5 mM potassium malate, 0.01 mM rotenone, 0.001 mM antimycin A, and 150 mM NH_4Cl (pH 7.4). The final volume in the cuvette was 2 ml. Depending on the experiment, 150 mM NaCl or 150 mM KCl was substituted for 150 mM NH_4Cl. The test compound (sulfluramid, DESFA or FCCP) was added 1 min prior to the RCM. RCM swelling was monitored by recording changes in absorbance at 520 nm at 25 °C over time. The change in absorbance from the first to the second minute was recorded as ΔA/min.

Mitochondrial proton movement. RCM proton movement was determined by the method of Reynafarje et al. [9]. Briefly, an aliquot of phosphate-depleted mitochondria (final concentration 2 mg protein/ml) was added to a temperature-regulated (25 or 37 °C) stirred cylinder containing a buffer (120 mM LiCl/10 mM KCl/3 mM Hepes (pH 7.2)), and pH was recorded over time. 2 min after the addition of RCM, the suspension was bubbled with O_2 for 2 min and valinomycin (100 ng/mg RCM protein) was added. 1 min later, 5 mM potassium pyruvate + 5 mM potassium malate were added. The test compound (sulfluramid, DESFA or FCCP) was added 0.5 to 0.8 min later.

Biochemical assays. To determine whether RCM metabolized sulfluramid, [*N-ethyl*-1-^{14}C]sulfluramid (14.1 mCi/mmol) (200 μM) was incubated with RCM in Q_{O_2} buffer containing 10 mM semicarbazide at 37 °C for 2 or 5 min. Boiled RCM served as a control. [*N-ethyl*-1-^{14}C]Sulfluramid was extracted according to the method of Thomas et al. [10]. DESFA formation was quantified from [^{14}C]acetaldehyde semicarbazone formation. Protein content was determined by the biuret method of Gornall et al. [11] after solubilization in 0.2 M NaOH/10% deoxycholate. Bovine serum albumin served as the protein standard.

Statistical analyses. The data are presented as the mean ± S.E. Each mitochondrial isolation represented an N of 1. Data were analyzed by analysis of variance or Student's t-test. Multiple means were tested for significance using Fisher's protected least significant difference test. A P value of less than 0.05 was considered significant.

Results

Both sulfluramid and DESFA increased state 4 respiration of RCM respiring on the site 1 substrates malate and pyruvate in a concentration-dependent manner (Fig. 1). The initial increase in state 4 respiration was seen with a concentration of 5 μM sulfluramid and 0.5 μM DESFA. Both compounds increased state 4 respiration to the same degree (120 nmol O_2/mg protein per min). The concentrations of sulfluramid and DESFA required to increase state 4 respiration by 50% were approx. 15 and 5 μM, respectively.

To compare the potency and efficacy of sulfluramid and DESFA to an agent known to increase state 4 respiration by uncoupling oxidative phosphorylation, FCCP was included in these studies (Fig. 1). While FCCP was as efficacious as sulfluramid and DESFA in increasing state 4 respiration, the concentration of FCCP needed to increase state 4 respiration by 50% was lower (0.1 μM). Thus FCCP was 150-times more potent than sulfluramid and 50-times more potent than DESFA in increasing state 4 respiration.

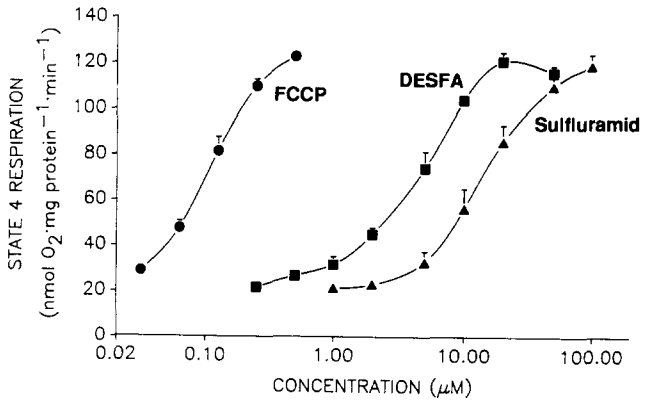

Fig. 1. The concentration-dependent effects of sulfluramid (▲), DESFA (■) and FCCP (●) on state 4 respiration of isolated rabbit renal cortical mitochondria respiring on pyruvate/malate (5 : 5 mM). Datum points are the mean ± S.E., N = 3–5. Lines were drawn by visual inspection.

TABLE I

The effect of DESFA, sulfluramid and FCCP on isolated rabbit renal cortical mitochondrial swelling in various solutions

See Methods for details. Data are $\bar{x} \pm$ S.E., $N = 3$–7. Values with different superscripts within a column are significantly different from one another ($P \leq 0.05$).

Treatment	Mitochondrial swelling (ΔA/min)		
	NaCl	NH_4Cl	KCl
Control	0.019 ± 0.002^{a}	0.024 ± 0.001^{a}	0.016 ± 0.002^{a}
DESFA (50 μM)	0.026 ± 0.003^{b}	0.042 ± 0.007^{b}	0.020 ± 0.004^{a}
DESFA (100 μM)	0.038 ± 0.003^{c}	0.062 ± 0.006^{c}	0.041 ± 0.005^{b}
Sulfluramid (200 μM)	0.017 ± 0.001^{a}	0.021 ± 0.003^{a}	0.016 ± 0.003^{a}
FCCP (0.5 μM)	0.030 ± 0.004^{b}	0.035 ± 0.002^{b}	0.022 ± 0.004^{a}
FCCP (1 μM)	0.027 ± 0.002^{b}	0.037 ± 0.004^{b}	0.024 ± 0.003^{c}

To determine whether the above effects were substrate-dependent, RCM respiring on succinate (in the presence of rotenone) were treated with 20 μM sulfluramid or 20 μM DESFA. State 4 respiration of control RCM was 59 ± 1 nmol O_2/mg protein per min, whereas sulfluramid and DESFA increased state 4 respiration to 136 ± 11 and 138 ± 18 nmol O_2/mg protein per min ($P \leq 0.05$), respectively.

To determine the effects of sulfluramid on state 4 respiration in the absence of proton movement via the F_0F_1-ATPase, RCM respiring on pyruvate and malate were exposed to sulfluramid in the presence and absence of oligomycin. Oligomycin is a specific inhibitor of F_0F_1-ATPase [12]. To ensure the inhibition of the F_0F_1-ATPase, ADP (0.4 μmol) was added prior to sulfluramid (100 μM) and had no effect. Sulfluramid increased state 4 respiration to the same degree in the presence (125 ± 10 nmol O_2/mg protein per min) or absence (113 ± 12 nmol O_2/mg protein per min) of oligomycin. Thus, the increase in state 4 respiration is not the result of proton movement via the F_0F_1-ATPase.

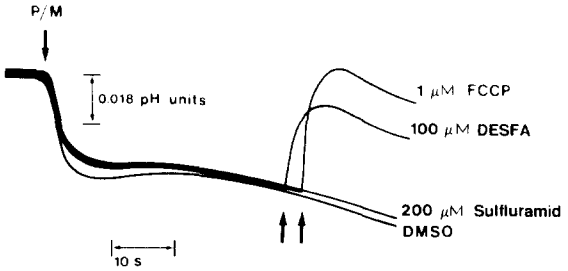

Fig. 2. The effect of sulfluramid, DESFA and FCCP on isolated rabbit renal cortical mitochondrial proton uptake. See Experimental procedures for details.

By measuring passive mitochondrial swelling in isotonic solutions containing various ions, RCM proton movement was determined indirectly. RCM did not swell when placed in a solution containing sucrose, but swelled rapidly in a solution containing ammonium acetate or sodium acetate (data not shown). These results are in complete agreement with those previously reported using rat liver mitochondria [7,8]. In the presence of DESFA (50 μM) or FCCP (0.5 μM), RCM swelled when placed in solutions containing NaCl and NH_4Cl but not KCl (Table I). Higher concentrations of DESFA (100 μM) and FCCP (1 μM) caused RCM to swell when placed in a solution of KCl. In contrast, sulfluramid (200 μM) did not cause RCM to swell when placed in a solution of NaCl, NH_4Cl or KCl. These results suggest that DESFA and FCCP, but not sulfluramid, increase RCM proton permeability.

The effect of sulfluramid, DESFA and FCCP on RCM proton permeability was also determined directly by monitoring the pH of RCM suspensions. The addition of pyruvate/malate to a suspension of RCM

TABLE II

The effect of perfluorooctane sulfonamide (DESFA), sulfluramid and fluorocyanocarbonylphenylhydrazone (FCCP) on isolated rabbit renal cortical mitochondrial proton uptake

Data are mean ± S.E. ($N = 4$–6). Values with different superscripts within a column are significantly different from one another ($P \leq 0.05$). n.d., not detected.

Treatment	Temp. (°C)	Proton ejection (ΔpH/mg per min)	Proton uptake (ΔpH/mg per min)	Proton uptake (ΔpH/mg)
Control	25		n.d.	n.d.
DESFA (100 μM)	25	0.036 ± 0.004^{a}	0.137 ± 0.010^{a}	0.012 ± 0.001^{a}
Sulfluramid (200 μM)	25		n.d.	n.d.
FCCP (1 μM)	25		0.219 ± 0.024^{b}	0.013 ± 0.001^{a}
Control	37		n.d.	n.d.
DESFA (100 μM)	37	0.101 ± 0.003^{b}	0.139 ± 0.031^{a}	0.005 ± 0.001^{b}
Sulfluramid (200 μM)	37		n.d.	n.d.
FCCP (1 μM)	37		0.264 ± 0.051^{b}	0.007 ± 0.001^{b}

resulted in a decrease in extramitochondrial pH, as the mitochondria actively pumped protons into the medium (Fig. 2). The addition of the vehicle DMSO or sulfluramid (200 μM) to RCM suspensions had no effect on extramitochondrial pH. However, the addition of DESFA (100 μM) or FCCP (1 μM) resulted in a rapid increase in extramitochondrial pH. The effects of sulfluramid, DESFA and FCCP on RCM proton uptake are summarized in Table II. While the extent of proton uptake by RCM was equivalent for DESFA and FCCP, the rate of proton uptake induced by FCCP was approximately twice that induced by DESFA. The rate of proton uptake was equivalent at 25 and 37°C for DESFA or FCCP. These results showed that DESFA and FCCP, but not sulfluramid, acted as protonophores in a temperature-independent manner. Furthermore, these experiments were in complete agreement with the RCM swelling experiments.

The observation that sulfluramid increased state 4 respiration but did not act as a protonophore suggested to us that sulfluramid might be biotransformed to DESFA by RCM and that DESFA might be responsible for the actions of sulfluramid. Following 2 min of incubation, RCM metabolized 200 μM sulfluramid to 2.2 ± 0.4 μM DESFA. After 5 min of incubation, the amount of DESFA formed increased to 4.3 ± 0.9 μM. Thus, RCM biotransform sulfluramid to DESFA in a time-dependent manner.

Discussion

The results of the present study show that sulfluramid and DESFA, independent of the metabolic substrate supplied, and the known uncoupler FCCP increased state 4 respiration of rabbit RCM to the same degree in the absence of a phosphate acceptor or in the presence of F_0F_1-ATPase inhibition. However, DESFA was 3-times more potent than sulfluramid as an uncoupler.

To determine whether the increase in state 4 respiration by sulfluramid and DESFA was associated with the dissipation of the RCM proton gradient, RCM proton movement was examined both indirectly and directly. Both DESFA and FCCP, but not sulfluramid, caused RCM to swell when placed in a solution containing NaCl and NH_4Cl, but not KCl. Likewise, DESFA and FCCP rapidly increased RCM proton uptake, as indicated by an increase in extramitochondrial pH, but sulfluramid had no effect on RCM proton uptake. These results show that DESFA, like the known protonophore FCCP, acts as a protonophore and may uncouple oxidative phosphorylation through this mechanism.

The observation that both sulfluramid and DESFA increased state 4 respiration but only DESFA increased RCM proton permeability raised the possibility that sulfluramid might be biotransformed to DESFA by RCM and that DESFA was responsible for the actions of sulfluramid. RCM were found to remove the *N*-ethyl group of sulfluramid rapidly and thus form DESFA. Furthermore, the amount of DESFA formed was consistent with the observed increases in state 4 respiration. The mitochondrial enzyme system responsible for the conversion of sulfluramid to DESFA is currently unknown, but may involve the mitochondrial cytochrome *P*-450-linked mixed function oxidase [13].

In general, chemicals which uncouple oxidative phosphorylation are lipophilic weak acids [12]. DESFA is a very lipophilic weak acid (pK_a of 9 in methanol) and could easily function as a shuttle for protons by combining and releasing a proton from the amino group. One possible explanation why sulfluramid did not act as a protonophore is the addition of the ethyl substituent to the amino group resulted in an increased pK_a (pK_a of 9.5 in methanol). Compared to other known compounds that uncouple oxidative phosphorylation [4,14,15], DESFA is structurally unique and is one of two uncouplers that does not contain a ring structure.

Both sulfluramid and DESFA are potent stimulators of basal and ouabain-insensitive Q_{O_2} in renal proximal tubules, with initial effects seen at concentrations of 5–10 μM [3]. DESFA was approx. 3-times more potent than sulfluramid in increasing basal and ouabain-insensitive Q_{O_2}. The ouabain-insensitive increases in basal Q_{O_2} elicited by sulfluramid and DESFA ruled out the possibility that sodium influx produced the increase in basal Q_{O_2} and raised the possibility that sulfluramid and DESFA might produce toxicity by uncoupling oxidative phosphorylation. The data obtained from this study completely support the hypothesis that the cellular effects and cytotoxicity of sulfluramid and DESFA are the result of uncoupling of oxidative phosphorylation by DESFA.

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