Esterification Prevents Induction of the Mitochondrial Permeability Transition by *N*-Acetyl Perfluorooctane Sulfonamides

Timothy M. O'Brien,*,† Robert M. Carlson,‡ Paulo J. Oliveira,§ and Kendall B. Wallace†

Department of Biochemistry and Molecular Biology, Toxicology Graduate Program, University of Minnesota, Medical School, 1035 University Drive, Duluth, Minnesota 55812, Department of Chemistry, University of Minnesota, 1035 University Drive, Duluth, Minnesota 55812, and Center for Neurosciences and Cell Biology, Department of Zoology, University of Coimbra, Coimbra, Portugal

N-Alkyl perfluorooctane sulfonamides have been widely used as surfactants on fabrics and papers, fire retardants, and anticorrosion agents, among many other commercial applications. The broad use, global distribution, and environmental persistence of these compounds has generated considerable interest regarding potentially toxic effects. We have previously reported that perfluorooctanesulfonamidoacetate (FOSAA) and *N*-ethylperfluorooctanesulfonamidoacetate (*N*-EtFOSAA) induce the mitochondrial permeability transition (MPT) in vitro, resulting in cytochrome *c* release, inhibition of respiration, and generation of reactive oxygen species. By synthesizing the corresponding methyl esters of FOSAA and *N*-EtFOSAA (methyl perlfuorinated sulfonamide acetates), we tested the hypothesis that the *N*-acetate moiety of FOSAA and *N*-EtFOSAA is the functional group responsible for induction of the MPT. Swelling of freshly isolated liver mitochondria from Sprague−Dawley rats was monitored spectrophotometrically and membrane potential ($\Delta\Psi$) was measured using a tetraphenylphosphonium-selective (TPP$^+$) electrode. In the presence of calcium, 40 μM FOSAA and 7 μM *N*-EtFOSAA each induced mitochondrial swelling and a biphasic depolarization of membrane potential. Mitochondrial swelling and the second-phase depolarization were inhibited by cyclosporin-A or the catalyst of K$^+$/H$^+$ exchange nigericin, whereas the first-phase depolarization was not affected by either. In contrast, the methyl esters of FOSAA and *N*-EtFOSAA exhibited no depolarizing or MPT inducing activity. Results of this investigation demonstrate that the carboxylic acid moiety of the *N*-acetates is the active functional group, which triggers the MPT by perfluorinated sulfonamides.

Introduction

Since the early 1950s, *N*-alkyl perfluorooctane sulfonamides, including *N*-acetyl-perfluorooctanesulfonamide (FOSAA)[1] and *N*-ethylperfluorooctanesulfonamidoacetate (*N*-EtFOSAA) have been widely used as surfactants on fabrics and papers, fire retardants, anticorrosion agents, and antistatic agents for film production, among other commercial applications (*1*). The perfluorooctane sulfonamides were manufactured for their chemical and thermal inertness and special surface-active characteristics. The high-energy fluorine−carbon bonds impart the physical and chemical stability that renders perfluorooctanes resistant to hydrolysis, photolysis, microbial degradation, and metabolism. Consequently, the perfluorooctanes are extremely persistent in the environment (*1, 2*).

The broad application, global distribution, and environmental persistence of these perfluorinated compounds has generated considerable concern regarding their potential toxicity (*3*). The terminal degradation product of most *N*-alkyl perfluorooctane sulfonamides is perfluorooctanesulfonate (PFOS). PFOS and FOSAA have been identified in individual and pooled human serum samples from the United States general population (*4*− *6*), and it has been shown that FOSAA and *N*-EtFOSAA are metabolites in the biotransformation pathway of perfluorinated sulfonamido alcohols (*7*). PFOS itself has been detected in birds, fish, and mammalian tissues from the Canadian Artic, Baltic and Mediterranean Seas, and the Great Lakes of North America (*1, 8*−*10*). Although the source of PFOS in human blood is not well-characterized, it may likely involve exposure to PFOS itself or precursor molecules such as FOSAA and *N*-EtFOSAA (*5*). Because there is much yet to be learned about the distribution and degradation of perfluorooctane sulfonamides, estimating environmental exposure to any particular perfluorooctane including FOSAA and *N*-EtFOSAA is complicated and inexact.

There have been a number of studies with a variety of exposure regimens in which a wasting syndrome or cachexia has been observed in animals exposed to perfluorinated acids. The most prominent acute and subchronic high-dose effects of perfluorinated acids in adult rodents and monkeys are altered lipid metabolism, hepatocellular hypertrophy, and vacuolation, decreased serum cholesterol, decreased triglycerides, hepatomegaly, decreased body weight, and death (*11*−*14*). PFOS exposure has also been shown to have adverse developmental effects in rodents where in vitro exposure results not only in maternal weight loss but also in increased abortions, reduced

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* To whom correspondence should be addressed. Phone: (218) 726-7927. Fax: (218) 726-8014. E-mail: obri0150@d.umn.edu.
† Department of Biochemistry and Molecular Biology, Toxicology Graduate Program, University of Minnesota, Medical School.
‡ Department of Chemistry, University of Minnesota.
§ University of Coimbra.
[1] Abbreviations: ANOVA, analysis of variance; ANT, adenine nucleotide translocator; BSA, bovine serum albumin; CsA, cyclosporin A; DMSO, dimethysulfoxide; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid; FOSAA, perfluorooctanesulfonamidoacetate; GC-ECD, gas chromatography with electron capture detection; MPT, mitochondrial permeability transition; *N*-EtFOSAA, *N*-ethylperfluorooctanesulfonami-doacetate; PFOS, perfluorooctanesulfonate; TPP$^+$, tetraphenylphosphonium ion; $\Delta\Psi$, mitochondrial membrane potential.

birth weights, and a profound neonatal morbidity in rats (*15*–*17*). In all of these studies the most prominent toxicological outcome appears to be a dose-dependent metabolic disruption. Inhibition of mitochondrial respiration and impairment of β-oxidation, as a result of uncoupling of oxidative phosphorylation or the induction of the mitochondrial permeability transition (MPT), have been implicated in a variety of pathologies related to disrupted lipid metabolism and hepatotoxicity (*18*). This suggests a potential role for mitochondrial dysfunction in the underlying mechanism of toxicity of the perfluorinated sulfonamides.

Multiple in vitro studies have shown the perfluorooctane sulfonamides to disrupt mitochondrial bioenergetics by three distinct mechanisms: uncoupling mitochondrial respiration, increasing nonspecific membrane permeability, and induction of calcium-dependent mitochondrial swelling (*19*–*21*). Specifically, FOSAA and *N*-EtFOSAA have been shown to be potent inducers of the MPT, which results in the release of cytochrome *c*, inhibition of respiration, and the generation of reactive oxygen species (*22*). The fact that these effects occur at low micromolar concentrations further supports the possibility that mitochondrial dysfunction may be an important factor in the pathogenesis caused by perfluorooctane sulfonamides.
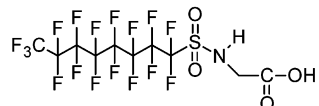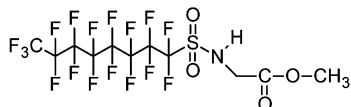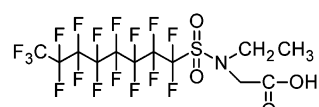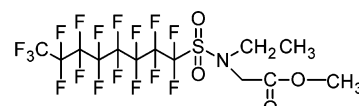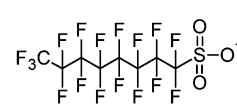
The mitochondrial permeability transition represents a non-selective increase in the permeability of the inner mitochondrial membrane to solutes up to 1.5 kD in size (*23*). It is characterized by dependence on matrix Ca^{2+} and is inhibited by elevated trans-membrane potential, acidic matrix pH, and the immunosuppressant cyclosporin-A (CsA) (*24*–*26*). Ca^{2+} can itself induce the MPT, but most often the MPT is a result of Ca^{2+} acting in conjunction with various "inducing agents" such as fatty acids or oxidants (*27*). Associated with MPT induction is the rapid equilibration of solutes across the mitochondrial membranes leading to depolarization of membrane potential, osmotic swelling, inhibition of oxidative phosphorylation, and release of potential apoptogenic factors, (*28*) including cytochrome *c* (*29*).

The purpose of the current investigation was to test the hypothesis that the *N*-acetate moiety of FOSAA and *N*-EtFOSAA is responsible for the induction of the MPT. To test this hypothesis, we synthesized methyl esters of the acetates and assessed them along with FOSAA and *N*-EtFOSAA for MPT inducing activity by measuring the change in $\Delta\Psi$ and mitochondrial swelling in the presence and absence of Ca^{2+}, nigericin, and/or CsA. The results reported herein add significant new understanding to the mechanism by which *N*-acetyl perfluorooctane sulfonamides induce the MPT and identifies the *N*-acetate as the critical functional group responsible for the activity of these compounds. The compounds chosen for inclusion in this investigation are depicted in Table 1.

Experimental Procedures

Mitochondrial Isolation. Mitochondria were isolated from liver of adult mixed-sex Sprague-Dawley rats (175–300 g body weight) by differential centrifugation (*30*). Rats were purchased from Harlan Sprague-Dawley (Madison, WI) and acclimated in an AAALAC-accredited, climate-controlled animal-care facility for at least 3 days and fasted for 12–18 h prior to the experiment. Animals were euthanized in a CO_2 chamber followed by decapitation. The liver was immediately excised, weighed, immersed in 40 mL of isolation medium (200 mM mannitol, 10 mM sucrose, 5 mM Hepes, 1 mM EGTA, pH 7.4) and minced with scissors. The mitochondria were isolated and prepared as previously described (*21*, *22*). The resulting mitochondrial pellet was re-suspended in a washing medium (200 mM mannitol, 10 mM sucrose, 5 mM Hepes, pH 7.4) to a final
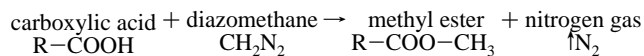
Table 1. Structures of the *N*-Acetylperfluorooctanesulfonamides and Corresponding Esters

protein concentration of 35–50 mg/mL. Protein concentration was determined with the Bradford assay using BSA as a standard (*31*).

Diazomethane Preparation. *p*-Toluenesulfonylmethylnitrosamide (Diazald, Aldrich Chemical Co., 4.28 g., 2 mmol) in ether (25 mL) was added slowly through a nonground glass separation funnel to a solution of potassium hydroxide (1.0 g) in water (2.0 mL) maintained at 65 °C in a nonground glass distillation apparatus (25 mL). As the reaction proceeded, the diazomethane co-distilled with ether into a receiving flask containing ether (10 mL). When all the Diazald was added, additional ether (10 mL) was introduced and distillation continued until the distillate was colorless. The resulting yellow solution of diazomethane in ether was used without further purification.

Ester Preparation. The ethereal solution of diazomethane was introduced dropwise into a solution of the fluorinated acids dissolved in ether using a fire-polished disposable pipet. The addition of diazomethane was continued until no further nitrogen gas was evolved and the resulting ester solution remained a pale yellow color. The ether and excess diazomethane was allowed to evaporate in a hood and the residual esters used without further purification.

$$\underset{\text{R–COOH}}{\text{carboxylic acid}} + \underset{\text{CH}_2\text{N}_2}{\text{diazomethane}} \rightarrow \underset{\text{R–COO–CH}_3}{\text{methyl ester}} + \underset{\uparrow\text{N}_2}{\text{nitrogen gas}}$$

The authors chose the diazomethane esterification method because of its specificity. This method is a widely used analytical protocol for synthesizing and quantifying small quantities of highly valuable carboxylic acids where purity and yield are critical. The U.S. EPA recognizes diazomethane esterification as a preferred method for methyl derivatization of organic halo-acid herbicides for GC quantification (U.S. EPA Method 8151a Revision − 1, 1996). Urbansky et al. used diazomethane esterification to demonstrate the quantitation of concentrations of halocarboxylates near the lower detection limits of gas chromatography with electron capture detection (GC-ECD) (*32*, *33*). Additionally, because analytical characterization of the perfluorinated test compounds is exceedingly

challenging and cost-prohibitive, we chose the method in which we could have the highest confidence in the purity and yield of the product.

Mitochondrial Swelling. Changes in mitochondrial volume were estimated by changes in light scattering as monitored spectrophotometrically at 540 nm (*21, 34*). Freshly isolated mitochondria were suspended at 0.5 mg/mL in 175 mM sucrose, 5 mM Hepes, 50 mM KCl, 2.5 mM KH$_2$PO$_4$, and 10 μM EGTA (pH 7.4) supplemented with 2 μM rotenone and 1 μg/mL oligomycin. The reaction was stirred continuously at 30 °C for 20 min. The mitochondria were energized with 5 mM succinate for 2 min before adding 25 μM CaCl$_2$. FOSAA, *N*-EtFOSAA, FOSAA methyl ester, or *N*-EtFOSAA methyl ester was added 2 min later. Where indicated, 2 μM of the MPT inhibitor cyclosporin A (CsA) (*24*) was added just prior to succinate, or 200 nM nigericin, a catalyst of K$^+$/H$^+$ exchange, was added just prior to the test compound. The criteria for establishing induction of the MPT was a decrease in light scattering of at least 0.5 absorbance units 6 min postaddition of test compound, which was inhibited completely by 2 μM CsA. O'Brien and Wallace previously established the concentrations of FOSAA and *N*-EtFOSAA used in this study (*22*). The phase partitioning properties of the perfluorinated octanes suggest that these compounds do not partition to the aqueous or organic phase, but rather to a separate phase altogether (*1*). However, in vivo, the perfluorinated octanes with poor aqueous solubility, including *n*-ethyl perfluoroctanesulfonamido ethyl alcohol, tend to partition to the proteins in the serum and liver (*35*). With solubility in mind the concentration of the methyl esters of FOSAA and *N*-EtFOSAA tested were 10 times that of the corresponding acetates, and all experiments were run in a continuously mixed reaction chamber at 30° to maximize ester partitioning to the mitochondrial fraction of the reaction.

TPP$^+$ Methods. A TPP$^+$ selective electrode was prepared as described by (*36*). Changes in membrane potential ($\Delta\Psi$) of rat liver mitochondria were measured by a tetraphenylphosphonium ion (TPP$^+$) sensitive electrode. Briefly, 0.5 mg of protein/mL was incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH$_2$PO$_4$, and 10 μM EGTA in the presence of 2 μM TPP$^+$, and supplemented with 2 μM rotenone and 1 μg/mL oligomycin. The reaction was stirred continuously at 30 °C during the incubation. The mitochondria were energized with 5 mM succinate for 1.5 min before adding 25 μM CaCl$_2$. FOSAA, *N*-EtFOSAA, FOSAA methyl ester, *N*-EtFOSAA methyl ester, or a DMSO control was added 2 min later. Where indicated, 2 μM of the MPT inhibitor cyclosporin A (CsA) or 200 nM nigericin was added just prior to the test compound. As reported, phase 1 depolarization describes the change in $\Delta\Psi$ immediately upon addition of the test compound, and phase 2 depolarization describes the change in $\Delta\Psi$ 6 min following the addition of the test compound. Results were reported in millivolts (mV), which were calculated as described (*36−38*). Through initial optimization we determined that 2 μM TPP$^+$ provided the most sensitive and responsive measure of $\Delta\Psi$ for this study.

Reagents. All fluorochemical compounds were synthesized, characterized, and provided gratis by the 3M Company, St. Paul, MN. The methyl esters of the fluorochemical acetates were synthesized in house. Cyclosporin A was provided as a generous gift of Sandoz Pharmaceuticals (East Hanover, NJ, www.sandoz.com), Ultra Pure sucrose was purchased from ICN Biomedicals, Inc. (Aurora, OH, www.icnbiomed.com), and all other reagents were from Sigma-Aldrich (St. Louis, MO, www.sigma-aldrich.com).

Hazardous Materials. Caution: *Diazald is a severe skin irritant and all skin contact should be avoided. Diazomethane is extremely toxic, is an irritant, and has been cited as a carcinogen (39−41). Diazomethane has been known to explode unaccountably both as a gas and in solution. Rough surfaces are proven initiators of detonation (41). Extreme caution should be used when handling both.*

Statistical Analysis. All experiments were repeated using freshly isolated hepatic mitochondria from at least three separate animals with a randomized selection of sexes. The results were analyzed
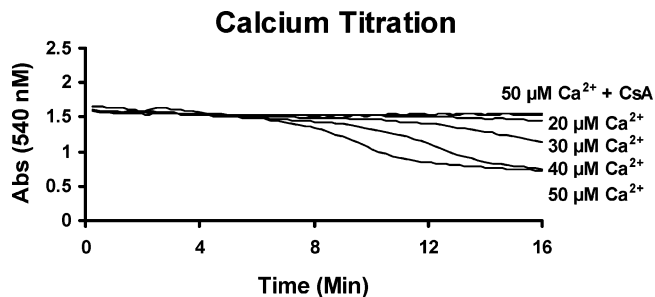
Figure 1. Calcium titration to measure permeability transition threshold. Swelling of rat liver mitochondria was monitored by light scattering at 540 nm. (0.5 mg of protein/mL) were incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH$_2$PO$_4$, 10 μM EGTA in the presence of 2 μM rotenone, 5 mM succinate, 1 μg/mL oligomycin, plus 0, 20, 30, 40, and 50 μM Ca^{2+}, and 50 μM Ca^{2+} plus cyclosporin A.

by 2-way ANOVA and Tukey's post-hoc test. A probability of (*p* < 0.05) was used as the criterion for statistical significance.

Results

Calcium Titration. Ca^{2+} is electrophoretically accumulated by mitochondria up to 1 μmol/mg of protein via the near 200 mV electronegative trans-membrane potential (*42*). However, there is a finite capacity which, if exceeded, triggers the mitochondria to undergo the induction of the mitochondrial permeability transition (MPT) (*23, 43*). In order to quantify calcium-loading capacity, which is considered to be a sensitive and specific indicator of mitochondrial competence and sensitivity to induction of the MPT (*21*), mitochondria were titrated with concentrations of calcium ranging from 0 to 100 nmol/mg of protein. Figure 1 is a representative tracing of a routine calcium titration. From this we established on average a concentration of 25 μM calcium (50 nmol/mg of protein) as an amount that is below the calcium loading capacity of control mitochondria, yet sufficient to support induction of the MPT in response to selected inducing agents.

Mitochondrial Membrane Potential ($\Delta\Psi$). Figures 2 and 3 illustrate a typical experiment for measuring the effects of the *N*-acetyl perfluorooctane sulfonamides (FOSAA and *N*-EtFOSAA, respectively) and the corresponding methyl esters on mitochondrial trans-membrane potential in the presence and absence of Ca^{2+}, nigericin, and CsA. The results indicate that there are two phases of depolarization that occur upon addition of FOSAA and *N*-EtFOSAA. The initial, partial depolarization occurs immediately upon addition of 40 μM FOSAA or 7 μM *N*-EtFOSAA (Figures 2 and 3; panels B, C, D, and E) where there is approximately a (+) (*15−25*) mV change in $\Delta\Psi$. This event occurs in the presence or absence of Ca^{2+}, nigericin, or CsA. In direct contrast, upon addition of the corresponding methyl esters of the acetates (Figures 2 and 3; panel F), there is no change in $\Delta\Psi$. Even at 10 times the concentration of the acetates, the esters still do not initiate depolarization or induce the MPT. The results of the initial depolarization for FOSAA, *N*-EtFOSAA in the presence and absence of Ca^{2+}, nigericin, and CsA, and the corresponding methyl esters are presented quantitatively in Figure 4, phase 1 depolarization. The initial depolarization induced by the DMSO control and the esterified treatments is significantly less than that of the FOSAA or *N*-EtFOSAA treatments in both cases.
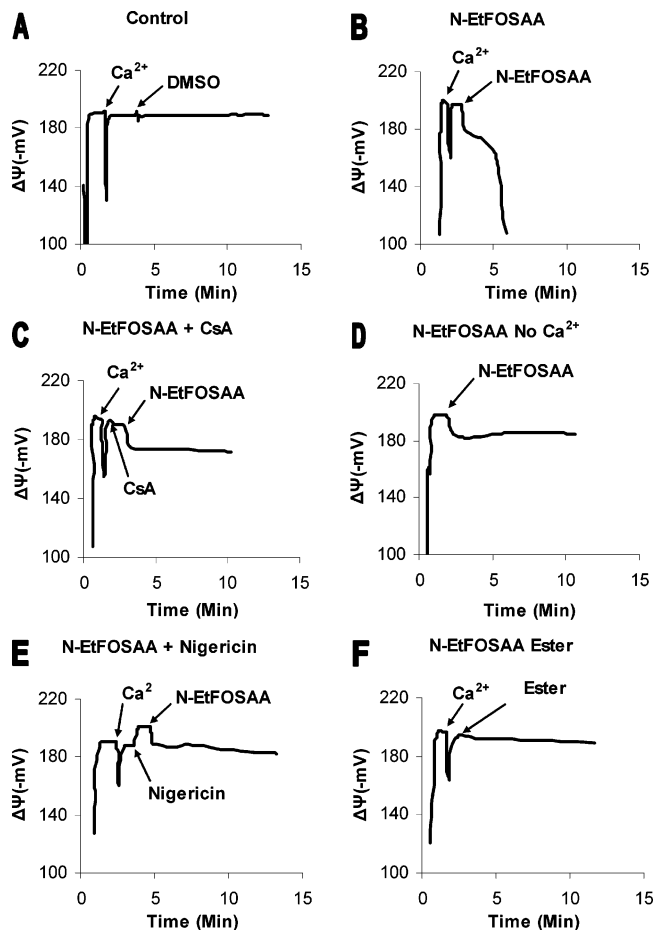
The slower phase of depolarization is reported as the change in $\Delta\Psi$ 6 min after the addition of the test compound. In Figures 2 and 3, panel B illustrates the change in $\Delta\Psi$ by FOSAA and *N*-EtFOSAA in the presence of Ca^{2+} absent nigericin and CsA.

1308 *Chem. Res. Toxicol., Vol. 19, No. 10, 2006*

O'Brien et al.
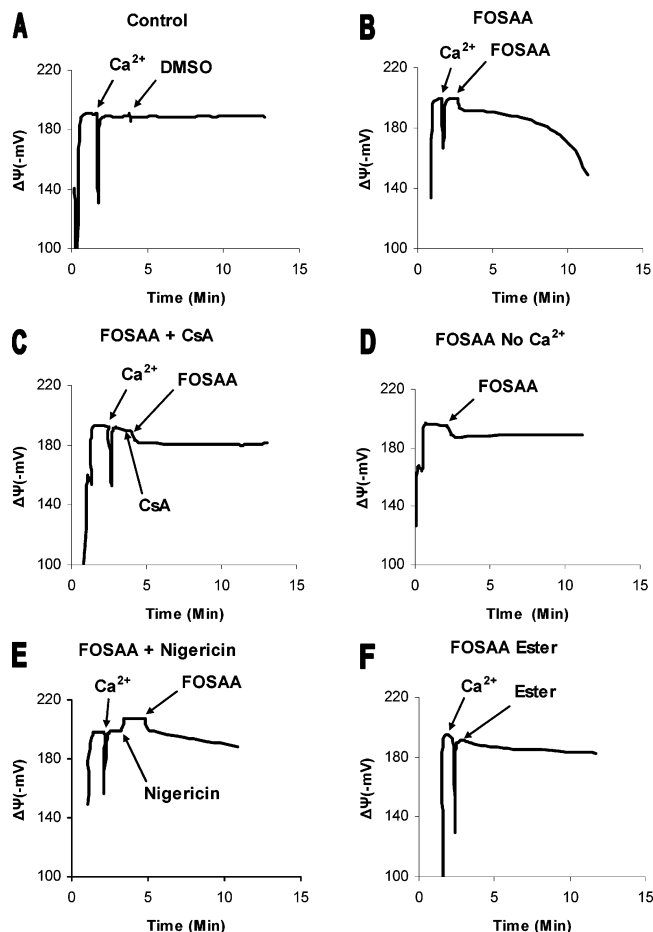
Figure 2. Membrane potential ($\Delta\Psi$) of rat liver mitochondria was measured by a tetraphenylphosphonium ion (TPP$^+$) sensitive electrode. Results reported in millivolts (mV). 0.5 mg of protein/mL were incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH$_2$PO$_4$, 10 μM EGTA in the presence of 2 μM TPP$^+$, 2 μM rotenone, 5 mM succinate, and 1 μg/mL oligomycin: (A) 25 μM Ca^{2+}, DMSO control (carrier); (B) 25 μM Ca^{2+}, 40 μM FOSAA; (C) 25 μM Ca^{2+}, 40 μM FOSAA, plus 2 μM cyclosporin A; (D) 40 μM FOSAA absent of Ca^{2+}; (E) 25 μM Ca^{2+}, 40 μM FOSAA, plus 200 nM nigericin; and (F) 25 μM Ca^{2+}, 400 μM FOSAA ester. Traces are representative of 3−5 repetitions, each using separate mitochondrial preparations.

Figure 3. Membrane potential ($\Delta\Psi$) of rat liver mitochondria was measured by a tetraphenylphosphonium ion (TPP$^+$) sensitive electrode. Results reported in millivolts (mV). 0.5 mg of protein/mL were incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH$_2$PO$_4$, 10 μM EGTA in the presence of 2 μM TPP$^+$, 2 μM rotenone, 5 mM succinate, and 1 μg/mL oligomycin: (A) 25 μM Ca^{2+}, DMSO control (carrier); (B) 25 μM Ca^{2+}, 7 μM N-EtFOSAA; (C) 25 μM Ca^{2+}, 7 μM N-EtFOSAA, plus 2 μM cyclosporin A; (D) 7 μM N-EtFOSAA absent Ca^{2+}; (E) 25 μM Ca^{2+}, 7 μM N-EtFOSAA, plus 200 nM nigericin; and (F) 25 μM Ca^{2+}, 70 μM N-EtFOSAA ester. Traces are representative of 3−5 repetitions, each using separate mitochondrial preparations.

This secondary depolarization does not occur in the absence of Ca^{2+} (Figures 2 and 3; panel D) and is inhibited in the presence of either CsA or nigericin (Figures 2 and 3; panels C and E, respectively). There is no occurrence of membrane depolarization in the presence of the DMSO control or the methyl esters of the acetates (Figures 2 and 3; panel F). The results of the secondary depolarization for FOSAA, N-EtFOSAA in the presence and absence of Ca^{2+}, nigericin and CsA, and the corresponding methyl esters are presented quantitatively in Figure 4, phase 2 depolarization. The secondary depolarization induced by the FOSAA or N-EtFOSAA + Ca^{2+} in both cases is significantly greater than that of the DMSO control. In all cases the absence of Ca^{2+}, the presence of nigericin, CsA, or the methyl esters of FOSAA and N-EtFOSAA significantly inhibited phase 2 depolarization caused by FOSAA and N-EtFOSAA plus Ca^{2+}.

Mitochondrial Swelling. Calcium-dependent, CsA-inhibited mitochondrial swelling measured by light scattering at 540 nM is a characteristic measure of MPT induction (25, 26). To verify that the observed membrane depolarization is related to the MPT induction, we recorded light scattering under the same treatment conditions as the membrane potential measurements. Figure 5

illustrates the typical experimental run for testing the effects of the N-acetyl perfluorooctane sulfonamides (FOSAA and N-EtFOSAA) and the corresponding methyl ester test compounds on mitochondrial light scattering at 540 nM in the presence and absence of nigericin or CsA. Figure 5, panels A and B, show that 40 μM FOSAA or 7 μM N-EtFOSAA induced mitochondrial swelling, both of which are inhibited by CsA. In contrast, 400 μM FOSAA methyl ester (Figure 5; panel A) and 70 μM N-EtFOSAA methyl ester do not induce mitochondrial swelling (Figure 5; panel B). Figure 5 panel C depicts nigericin inhibition of mitochondrial swelling. Two hundred nanomolar nigericin in a buffer containing 51 mM K$^+$ protects against mitochondrial swelling induced by FOSAA and N-EtFOSAA. In all cases there is a delay from the time of test compound addition until the initiation of mitochondrial swelling.

The results of mitochondrial swelling induced by FOSAA, N-EtFOSAA in the presence of Ca^{2+}, plus or minus nigericin or CsA, and the corresponding methyl esters are presented quantitatively in Figure 5, panel D. In each case there is a significant decrease in light scattering by FOSAA and N-EtFOSAA versus control, whereas the absence of Ca^{2+} or in the presence of nigericin or CsA protects against mitochondrial
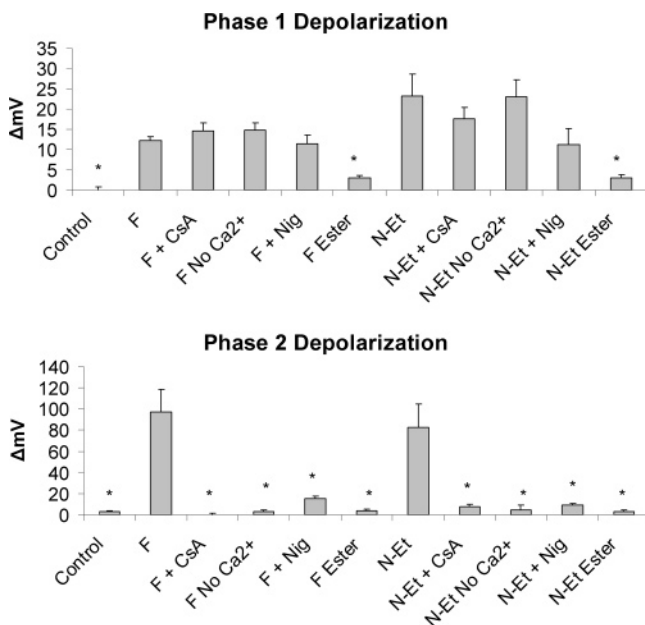
Figure 4. F (FOSAA) and *N*-Et (*N*-EtFOSAA) induced change in membrane potential ($\Delta\Psi$) as measured by a (TPP$^+$) sensitive electrode. Results reported in millivolts (mV). **Phase 1 depolarization** is the immediate change in $\Delta\Psi$ upon addition of the test compound. **Phase 2 depolarization** is the change in $\Delta\Psi$ 6 min after the addition of the test compound. The concentrations and additions are as reported in Figures 2 and 3. Bars represent the mean $\pm$ S.E.M. of 3−5 repetitions each analyzed by 2-way ANOVA and Tukey's post-hoc test. A probability of ($p < 0.05$) was used as the criterion for statistical significance. (*) Statistically significant difference compared to treatment alone.

swelling. The change in light scattering caused by the methyl esters was significantly less than that induced by FOSAA and *N*-EtFOSAA plus Ca^{2+}. This data indicates that swelling is directly related to induction of the MPT by the *N*-acetyl perfluorooctane sulfonamides and that the *N*-acetate of FOSAA and *N*-EtFOSAA is the critical functional group responsible for the activity of these compounds.

Discussion

The results of this investigation demonstrate that induction of the MPT by the *N*-acetyl perfluorooctane sulfonamides, FOSAA and N-EtFOSAA, is an event that is entirely dependent upon the carboxylic acid functional group. Induction of the MPT is preceded by a partial (15−25 mV) depolarization of $\Delta\Psi$ that occurs immediately upon addition of the *N*-acetyl test compounds (Figures 4; phase 1 depolarization). This initial depolarization occurs in the absence of Ca^{2+} and in the presence of cyclosporin-A, indicating that it is not a result of MPT induction. The fact that the initial depolarization is completely blocked by methyl-esterification of the acids, but not by nigericin, is evidence that the effect requires the carboxyl moiety and appears to be the result of an immediate translocation of charge across the inner mitochondrial membrane. This electrophoretic interpretation of the TPP$^+$ data is supported by the fact that in the presence of CsA there is no swelling (Figure 5), whereas nonspecific swelling would be expected if the initial depolarization were the result of generalized membrane dissolution by the perfluorinated sulfonamides.

The second component of membrane depolarization is the gradual and complete collapse of $\Delta\Psi$ (Figures 2 and 3, panel B). This secondary phase requires Ca^{2+} and is inhibited by CsA or nigericin, indicating that it is a consequence of MPT

induction. The secondary-phase depolarization does not occur with the corresponding methyl esters of the acetates, providing further evidence that mitochondrial membrane depolarization and induction of the MPT are entirely dependent upon the presence of the carboxylic acid.

The methyl esters of the acetates used in this investigation do not induce mitochondrial swelling (Figure 5A,B), nor do they cause membrane depolarization as is observed with FOSAA and *N*-EtFOSAA. Membrane integrity and membrane potential are not compromised in the presence of the esters; therefore, general membrane solubility of the perfluorinated compounds is likely not the disruptive mechanism observed in this investigation. These findings are in agreement with those of Starkov and Wallace who found that the perfluorinated compounds without amide or acidic moieties do not disrupt mitochondrial bioenergetics (*21*). Under every treatment condition involving FOSAA and *N*-EtFOSAA the initial depolarization occurs immediately upon addition, yet only in the presence of Ca^{2+} does the secondary depolarization occur. This evidence suggests a specific interaction with mitochondrial targets leading to an immediate depolarization of membrane potential, which does not occur in the absence of the carboxylic acid moiety.

The relationship between mitochondrial depolarization and MPT induction has been the subject of many studies. It has been demonstrated that at low membrane potential mitochondrial redox potential is decreased because NAD(P)H transhydrogenase cannot sustain adequate NADPH and GSH levels (*44*), which may result in MPT induction. In fact, Castilho et al. proposed that a decrease in $\Delta\Psi$ results in Ca^{2+}-induced ROS accumulation, thiol cross-linking, and MPT induction (*45*). Others advocate that the MPT is voltage-sensitive and a decrease in $\Delta\Psi$ results in a greater likelihood of MPT induction (*25*, *46*). Regardless of the initiating mechanism, the final outcome of MPT induction is a complete collapse of the $\Delta\Psi$ and, consequently, the electrochemical proton gradient (*47*).

There have been a multitude of studies regarding the mechanism by which fatty acids induce the MPT. It has been reported that fatty acid induced MPT is a result of proton translocation across the inner mitochondrial membrane which lowers $\Delta\Psi$ to a MPT gating potential (*27*, *48*). Once the gating potential is achieved, MPT induction is more likely. Other proposed mechanisms include the cycling of monovalent cations other than protons caused by fatty acid membrane translocation, again resulting in an electrogenic triggering of mitochondrial swelling (*49*). These mechanisms appear to require the ionized fatty acid be shuttled back to the inner membrane space through a process that apparently involves transporters such as the adenine nucleotide translocator (ANT) (*50*) or dicarboxylate carrier (*51*, *52*). Regardless of the specific target, each proposed mechanism has in common a process by which fatty acids "flip-flop" back and forth across the inner mitochondrial membrane and cause a dissipation of $\Delta\Psi$. Although the perfluorinated carbon tail of FOSAA and *N*-EtFOSAA imparts a more rigid physical character to the molecule, the aliphatic head of the sulfonamide will have properties comparable to non-fluorinated fatty acids. It is possible that the *N*-acetyl perfluorinated sulfonamides interact with the inner mitochondrial membrane in a fashion similar to non-fluorinated fatty acids.

The findings of this investigation allow us to propose that the initial depolarization of mitochondrial $\Delta\Psi$ is an electrophoretic event caused by the *N*-acetyl perfluorooctane sulfonamides translocating charge, most likely protons, across the mitochondrial membranes. The specific mechanism of the observed charge translocation is not completely clear. It may
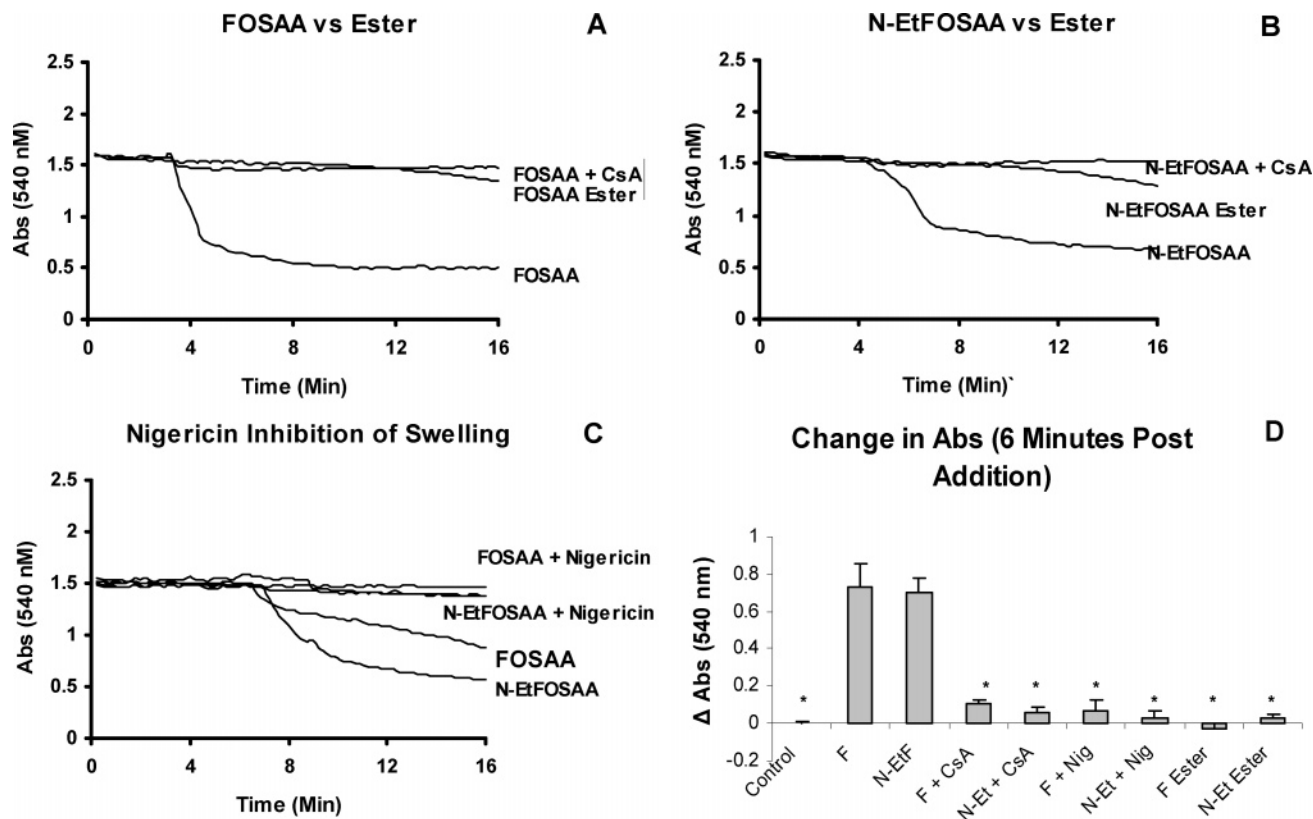
Figure 5. Swelling of rat liver mitochondria was monitored by light scattering at 540 nm. (0.5 mg of protein/mL) were incubated at 30 °C in a buffered solution of 175 mM sucrose, 50 mM KCl, 10 mM MOPS, 1 mM KH_2PO_4, 10 μM EGTA in the presence of 2 μM rotenone, 5 mM succinate, 1 μg/mL oligomycin, 25 μM Ca^{2+} plus the selected perfluorinated compound or DMSO (carrier) at the indicated concentration in the presence and absence of 2 μM cyclosporin A (CsA). Controls were treated with 25 μM Ca^{2+}. Mitochondrial swelling traces are representative of 3 repetitions, each using separate mitochondrial preparations. (A) 400 μM FOSAA ± cyclosporin A, 400 M FOSAA ester; (B) 7 μM *N*-Et-FOSAA ± cyclosporin A, 70 μM *N*-EtFOSAA ester; (C) 7 μM *N*-EtFOSAA, 40 μM FOSAA in the presence and absence of 200 nM nigericin; (D) F (FOSAA) and *N*-Et (*N*-EtFOSAA) induced decrease in absorbance measured at 540 nM. Results reported as change in arbitrary absorbance units 6 min after the addition of the test compound. Bars represent the mean ± S.E.M. of 3 repetitions each analyzed by 2-way ANOVA and Tukey's post-hoc test. A probability of ($p < 0.05$) was used as the criterion for statistical significance. (*) Statistically significant difference compared to the treatment.

be the result of a rearrangement of membrane phospholipids, which induces a generalized proton leak, or it could be due to direct protonophoric activity of FOSAA and *N*-EtFOSAA. Calculated pK_a values of less than 5 for the test compounds would support the first interpretation, but the fact that the esters do not induce phase 1 depolarization, or that there is no mitochondrial swelling induced by FOSAA or *N*-EtFOSAA in the presence of CsA, supports the protonophoric interpretation. In either case, as a consequence of phase 1 depolarization, $\Delta\Psi$ is depressed, causing the mitochondria to become more susceptible to MPT induction. The hyperpolarization and matrix acidification induced by the presence of nigericin (*25*, *53*), which inhibits the MPT, also supports this interpretation. The secondary membrane depolarization observed is specifically a consequence of MPT induction. The results also provide insight as to why the fully substituted sulfonamide *N*-EtFOSAA is approximately 5 times more potent than the secondary substituted sulfonamide FOSAA. It is estimated that the pK_a of the proton on the secondary substituted sulfonamide is quite low due to its proximity to the strong electron-withdrawing effects of fluorine, and therefore, the secondary amide of FOSAA would be present in its anionic state under the conditions of these experiments. This anionic state would render FOSAA less soluble in the mitochondrial membranes, whereas the fully substituted sulfonamide *N*-EtFOSAA does not have that anionic site and therefore is suspected to be much more membrane-soluble.

In conclusion, the fact that the pathologies expressed in vivo resulting from exposure to perfluorinated acids in rodents and

monkeys are altered lipid metabolism, hepatocellular hypertrophy and vacuolation, decreased serum cholesterol, decreased triglycerides, hepatomegaly, and decreased body weight with no obvious histopathology provides strong evidence that these events are metabolic in nature. PFOS has generated considerable interest because of its environmental penetrance and persistence. It has been detected in a variety of species of wildlife worldwide with mean liver concentrations as high as 1.7 ppm in predators such as polar bears (*9*). Exposure of the general human population is demonstrated by the detection of PFOS in banked serum at concentrations of 0.03−0.175 ppm (*4*, *6*, *54*) with occupational exposures of fluorochemical production workers reported at 0.04−12.70 ppm serum PFOS (*55*). Since PFOS has been suggested to be the terminal metabolite of the *N*-alkyl-perfluorooctanesulfonamides in vivo (*3*, *14*, *17*), the detection of PFOS in human or environmental monitoring studies may reflect either direct exposure to PFOS itself or exposures to more complex structural analogs. The concentrations used in this study are higher than those currently observed in animal or human serum samples (FOSAA, approximately 22 ppm, and *N*-EtFOSAA, approximately 3.2 ppm compared to the PFOS concentration of 12 ppm in humans). However, the in vivo partitioning of the perfluorinated sulfonamides to proteins in the liver and serum, and the fact that FOSAA and *N*-EtFOSAA induce acute in vitro outcomes resulting in a distinct disruption of mitochondrial bioenergetics that may be related to the general metabolic disruption observed in vivo, supports the relevance of the concentrations applied. More importantly, the concentra-

tions of PFOS observed in serum and liver of neonates with high mortality in a PFOS toxicity study (*56*) exceed the concentrations of *N*-EtFOSAA used in this study by more than 10-fold. Finally, identification of the carboxylic acid moiety as requisite to initiate the MPT adds significant new understanding to the process by which *N*-acetyl perfluorooctane sulfonamides disrupt mitochondrial bioenergetics and supports the hypothesis that this disruption plays a significant role in the observed pathological outcomes in vivo.

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