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Sequestration of coenzyme A by the industrial surfactant, Toximul MP8 A possible role in the inhibition of fatty-acid β-oxidation in a surfactant/influenza B virus mouse model for acute hepatic encephalopathy

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Abstract

We have investigated the mechanistic basis of our recent observation that exposing young mice to an industrial surfactant potentiates the inhibition of fatty-acid β-oxidation that occurs with subsequent virus infection (Murphy et al., Biochim. Biophys. Acta 1315, 208–216, 1996). In our mouse model for acute hepatic encephalopathy (AHE), neonatal mice were painted on their abdomens from birth to postnatal day 12 with nontoxic amounts of the industrial surfactant, Toximul MP8 (Tox), and then infected with a sublethal dose (LD_{30}) of mouse-adapted human Influenza B (Lee) virus (FluB). Mortality in mice treated with Tox + FluB was significantly higher than that in mice treated with FluB alone. In vitro assays of hepatic β-oxidation of [1-^{14}C]palmitic and [1-^{14}C]octanoic acids in the presence or absence of exogenous coenzyme A (CoA) indicated that Tox-mediated inhibition of oxidation was masked when CoA was added to the assays. FluB also inhibited β-oxidation by 20–30%, however this effect was independent of exogenous CoA which suggested that it involved a different mechanism. Tox-mediated potentiation of the inhibitory effect was most obvious (> 80% inhibition) when assays were done without added CoA. Analysis of hepatic CoA and its esters indicated that levels of both free CoA and acetyl-CoA were significantly lower in mice that were painted with Tox for 12 days. Tox-dependent reductions of acetyl-CoA were transient and returned to normal values after cessation of painting, whereas those of CoA persisted. FluB infection alone significantly reduced hepatic acetyl-CoA and the magnitude of this reduction (> 30%) was not affected by pre-exposing the mice to Tox. Relative to control mice, levels of acid insoluble acyl-CoA esters were elevated significantly in FluB and Tox + FluB treated mice. Activation of both [1-^{14}C]palmitic and [1-^{14}C]octanoic acids was reduced in Tox-exposed mice at experimental day 12, but only when exogenous CoA was not included in the assay media; this effect appeared to persist after cessation of painting. Collectively, these data support the concept that Tox and FluB have independent effects on hepatic CoA metabolism that are associated with abnormalities in fatty-acid β-oxidation. However,

Abbreviations: AHE, acute hepatic encephalopathy; Tox, Toximul MP8; FluB, mouse-adapted human influenza B (Lee) virus; RS, Reye's Syndrome; CoA, coenzyme A; MEM, minimal essential medium; PCA, perchloric acid; WSP, water-soluble product(s); BSA, bovine serum albumin.

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these do not fully explain the synergistic effect of the virus and chemical on β-oxidation inhibition, which is a candidate co-mechanism for potentiation of mortality in this mouse model of AHE.

Keywords: Acute hepatic encephalopathy; Surfactant/virus mouse model; Hepatic fatty acid β-oxidation; Coenzyme A metabolism; Reye's Syndrome; Fatty acid activation

1. Introduction

Disturbances in hepatic fatty-acid β-oxidation are being described more frequently with clinical toxicity in association with an increasing number of structurally-diverse xenobiotic chemicals (for review, see [1,2]), including therapeutic agents such as salicylates [3,4], antibiotics [5], valproic acid [6], and known toxins such as 3-mercaptopropionic acid [7] and hypoglycin [8]. These appear to function by a variety of different mechanisms, but the resulting inhibition of this crucial pathway in intermediary metabolism results in hepatotoxicity leading often to coma and death.

For many years we have used a surfactant/virus mouse model to study Reye's Syndrome (RS), a frequently-cited example of AHE. The model involves painting neonatal mice with apparently nontoxic amounts of the industrial surfactant which is frequently used in forestry spray programs, Toximul MP8 (Tox), on their abdomens daily for 12 days, and subsequently infecting them with sublethal doses (LD_{30}) of mouse-adapted human influenza B (Lee) virus (FluB). Our studies demonstrated that Tox + FluB treatment produces many of the features of human RS, including mitochondrial structural changes, hyperammonemia, elevated intracranial pressure and a high mortality rate [9–13]. Recently, we reported that Tox exacerbates virus-induced inhibition of fatty-acid β-oxidation in the mouse model [14]. These initial in vitro assays with liver homogenates from the different treatment groups (control, Tox, FluB and Tox + FluB) indicated an apparent time-dependent increase in oxidation of [1-^{14}C]palmitic acid in mice painted with Tox, inhibition of its oxidation associated with FluB infection, and a potentiation of the inhibitory effect in mice given combined Tox + FluB treatment. Other than having indirect evidence that treatment with Tox alone stimulated peroxisomal oxidation, we did not investigate the mechanisms underlying these observations, nor did we resolve the apparent discrepancy between the effects of Tox, FluB and Tox + FluB.

Recent reports that some of the xenobiotics could be inhibiting β-oxidation by sequestering or depleting intracellular CoA suggested a possible explanation for some of our above findings. This phenomenon was demonstrated with valproic [15,16] and salicylic acid [3], ibuprofen [17] and pivampicillin [18]. Each of these agents is either a carboxylic acid or is converted into one in vivo, and subsequently activated to a CoA thioester. As such, they have the potential to compete with endogenous carboxylic acids for the CoA that is necessary to undergo key metabolic reactions, including β-oxidation. The Tox that we use in the mouse model is a complex mixture of anionic and nonionic hydrocarbons [19], some of which are converted by ω-oxidation to carboxylic acids in vivo, activated to acyl-CoA esters, and degraded in the peroxisomes by β-oxidation [20]. Therefore, we postulated that Tox also could be sequestering hepatic CoA, and that this would account for its ability to potentiate FluB-dependent inhibition of β-oxidation. Such an action in earlier studies would not have been apparent, as these in vitro experiments were carried out in the presence of saturating quantities of all reaction cofactors, including CoA. The purpose of the present study was to determine how presumably nontoxic doses of Tox and infection with FluB affect cellular CoA metabolism, including levels of CoA and its metabolites, and such processes as fatty-acid activation to acyl-CoA esters by acid:CoA ligase (EC 6.2.1.1-3).

2. Materials and methods

2.1. Materials

[1-^{14}C]Palmitic acid (specific activity, 56 mCi/mmol) and [1-^{14}C]octanoic acid (specific activity, 53.5 mCi/mmol) were obtained from Dupont

Canada (Mississauga, Canada). Palmitic acid and other lipid standards were from Serdary Research Laboratories Inc. (London, Canada). Phospho-transacetylase (PTA, EC 2.3.1.8), acetylphosphate, citrate synthase, ATP, L-carnitine, coenzyme A, dithiothreitol and fatty-acid-poor bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Whatman glass microfibre paper (GF/B) was from Fisher Scientific Co. (Halifax, Canada). FluB was originally obtained from Dr. Frank Ennis, National Institutes of Health, Bethesda, MD. Toximul MP8 (Tox, Lot #9-30162) was from Charles Tenant Co., Toronto, Canada.

2.2. Surfactant / FluB virus mouse model

The surfactant/virus mouse model for AHE has been described in detail elsewhere [11,14]. Briefly, male and female Swiss Webster (ND4, Harlan Sprague Dawley, Indianapolis, IN) white mice were mated and the mothers were allowed to deliver. Mice born on the day in which the largest number of births occurred were pooled and were randomly divided into groups of nine pups per nursing mother. 24 h later, the pups were divided arbitrarily into two groups; minimal essential medium (MEM) was applied dermally to the abdomens of the first group, while the other group was painted with the industrial surfactant, Tox, that was diluted in MEM (1%, by vol). This treatment was repeated daily for 12 days. For the first 5 days, the mice received 8.6 ± 2.6 mg Tox/day; the dosage was increased to 25.8 ± 9.0 mg/day for the last 7 days. On day 14, half of each of the MEM and Tox groups received 10 μl of mouse-adapted human influenza B (Lee) virus (FluB) (640 HA U/0.5 ml) intranasally under light ether anaesthesia; the other half of each group received anaesthetic alone. Deaths were recorded each morning throughout the experiment. All studies were carried out in accordance with the guidelines set by the Canadian Council on Animal Care.

2.3. Fatty-acid β-oxidation assay

At different times during the painting and post-infection periods, 3 to 6 mice were taken from each experimental group and were killed by decapitation. The livers were excised immediately in ice-cold modified Krebs-Henseleit bicarbonate buffer (pH 7.4) that contained no Ca^{2+} and 2 mM Mg^{2+} and was gassed with CO_2/O_2 (95/5, v/v) for at least 20 min. Homogenates (1–5%, wt/vol) were prepared using 6 strokes of a glass-Teflon homogenizer, and were used immediately for the assays, as β-oxidation activity is not detected in frozen tissue. [1-^{14}C]Palmitic acid β-oxidation was measured essentially as described previously [14]. The reaction mixtures contained, in a final volume of 150 μl of modified Krebs-Henseleit buffer: 0.2 mM [1-^{14}C]palmitic acid (4000–5000 dpm/nmol) bound to fatty-acid poor BSA in a 3:1 molar ratio, 1 mM ATP, 2 mM dithiothreitol, 0.5 mM L-carnitine, with or without 0.05 mM CoA. The reaction mixture was pipetted into the bottom of a 1.5 ml polypropylene tube that was placed in a 20 ml screw-cap glass liquid scintillation vial. An 8 mm disk of GF/B glass fibre paper that was saturated with 80 μl of hyamine hydroxide (1 M) in methanol was placed on the bottom of the glass vial away from the reaction tube. Reactions were initiated by addition of 50 μl of liver homogenate (30–50 μg protein when CoA was added to the mixture, or 150–160 mg protein when there was no exogenous CoA), and incubations were continued for 5 min at 37°C. We had determined previously that these parameters were optimal for the in vitro assay. Reactions were terminated by the addition of 20 μl of perchloric acid (PCA, 70%, by vol) to the assay tube, and the vials were quickly recapped and allowed to equilibrate for 30 min. The tubes were removed from the vials, 7 ml of scintillation fluid were added to the latter, and radioactivity in $^{14}CO_2$ was counted. The contents of the polypropylene tubes were extracted with chloroform/methanol (2/1, vol/vol), and samples of the aqueous and organic phases were counted for measurement of water-soluble (WSP) and lipid products, respectively. Rates of β-oxidation were taken as the sum of $^{14}CO_2$ + ^{4}C-WSP formed/mg homogenate protein. Protein was determined using the method of Lowry et al. [21], using BSA as the standard.

Assays for oxidation of [1-^{14}C]octanoic acid were carried out using essentially the same procedure, with 60–80 mg protein/tube. After the reactions were terminated, chloroform/methanol (2/1, v/v) was added to the reaction mixture to form two phases. The aqueous phases (0.4 ml) were extracted twice with 1.33 ml of *n*-hexane. This has been demon-

strated to remove $>98\%$ of [1-^{14}C]octanoic acid without removing ketone bodies or other WSP [3,22]. Aliquots of the aqueous phases were counted for ^{14}C-WSP.

2.4. Quantitation of hepatic CoA and CoA esters

At select times during the experiment, 4 to 6 mice from each experimental group were killed by decapitation and the livers were frozen immediately in liquid nitrogen. At the time of sampling, portions of the livers were weighed and homogenized in 0.6 N PCA. The mixtures were centrifuged, and the supernatants transferred to a second set of tubes, adjusted to pH 4 with 2 M $KHCO_3$, and analyzed for CoA and its esters using the enzyme cycling method described by McDougal and Dargar [23]. This method is based on the CoA-dependent disappearance of acetylphosphate (AcP) when tissue extracts are incubated with phosphoacetyltransferase (PTA; EC 2.3.1.8). To quantitate total free CoA plus acid-soluble CoA esters, the supernatants were heated for 30 min at 60°C (pH 9.6) in the presence of 6 mM dithiothreitol (DTT). After being readjusted to pH 4.5, aliquots were incubated with arsenate buffer (120 mM, pH 7.8), and recycling reagent (15 mM AcP + 75 mg/ml PTA). After 17–20 h, the tube contents were reacted with hydroxylamine and $FeCl_3$, and the color product was quantitated spectrophotometrically. The relationship between the amount of AcP remaining and CoA (plus ester) concentration in the samples was determined by comparison with values obtained for a parallel set of CoA standards.

Levels of free CoA and acetyl-CoA were determined indirectly by omitting the DTT treatment and altering incubation conditions in such a way that specific components were released from the tissue extract and destroyed by oxidation by reaction with 0.3% H_2O_2 and 12 mM $CuSO_4$. The remaining esters were then quantitated using the cycling reaction described above. The sum of acetyl-CoA, acetoacetyl-CoA plus other PTA-reactive species were determined using the cycling reaction after CoASH and succinyl-CoA were oxidized following their selective liberation by heating the samples at 85°C for 15 min. Acetyl-CoA was then quantitated as its loss upon heating the samples (85°C, 15 min) in the presence of citrate synthase (5 mg/ml; EC 4.1.3.7) and oxaloacetate. Succinyl-CoA was measured in isolation after free CoA and all esters were converted to CoASH (0°C, 15 min, 40 mg/ml PTA) and oxidized with the cycling reagent that was supplemented with $MgCl_2$, GDP and succinic thiokinase (EC 6.2.1.4). Appropriate blanks were used for all of the assays. Quantities of individual CoA species were calculated by subtracting the amounts of other components from the total amounts of CoA obtained in the assays with DTT.

Quantitation of the levels of acid-insoluble long-chain fatty acyl-CoA esters involved PCA precipitates from the initial centrifugation being washed and dispersed in 75 mM 2-methyl,2-amino,propan-1-ol that contained 6 mM DTT. Mixture preparation included adjusting the pH to 9.3, heating at 60°C for 30 min, reacidification, and centrifugation. The supernatant was treated with DTT and carried through the cycling reaction as described above.

2.5. Measurement of fatty-acid: CoA ligase activities

The liver homogenates were assayed for fatty acid:CoA ligase activity as described by Singh and Poulos [24], with minor modifications. The reaction mixtures contained in a final volume of 0.2 ml of modified Krebs-Henseleit buffer (pH 8.0): 0.2 mM [1-^{14}C]palmitic or [1-^{14}C]octanoic acid, 1 mM ATP, 2 mM DTT, 2 mM KCN, and where indicated, 0.05 mM CoA. Reactions were initiated by addition of 30–50 mg of protein when exogenous CoA was present or 150–160 mg when it was omitted, and were continued at 37°C for 5 min. They were terminated with 20 μl of 10 M HCl, and 1.5 ml of an extraction mixture (isopropanol:heptane:10 M HCl, 40:10:1, by vol) was added. The resulting two phases were separated by the addition of 1.5 ml heptane and 1 ml water. Aqueous phases were washed twice with 2 ml of heptane and aliquots were counted to quantitate the amount of [1-^{14}C]fatty acyl-CoA formed. Blank values were obtained by mixing all reactants and terminating the reaction at $t = 0$.

2.6. Statistical analyses

The data represent means $\pm$ SD of values obtained from 3–6 different mice. They were analyzed using a Student's unpaired *t*-test with Bonfarroni correction

for multiple testing. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Mortality

In the surfactant/FluB mouse model of AHE, mice infected with FluB, with or without pre-exposure to Tox generally begin to die two days after inoculation (i.e., experimental days 15, 16), and the maximum number of deaths is established between Days 18 and 21. On day 19 in a representative experiment [14], the % mortality in the FluB and Tox + FluB groups was 42% and 78%, respectively.

3.2. Effects of exogenous CoA on Tox and/or FluB-mediated alterations of hepatic β-oxidation

The first question that we addressed in investigating the mechanism(s) underlying the alterations in hepatic β-oxidation in the mouse model (i.e., enhancement by dermal Tox, inhibition by FluB infection) was whether these alterations were affected by the availability of CoA. Parallel assays of β-oxidation of [1-^{14} C]palmitic acid with and without exogenous CoA were carried out with homogenates of livers from mice at experimental days 12 (MEM, Tox) and 19 (MEM, Tox, FluB, Tox + FluB). Without CoA added, the maximum oxidation rates with this fatty acid were only about 5% of those when CoA was present in the assay media (Fig. 1). Never-

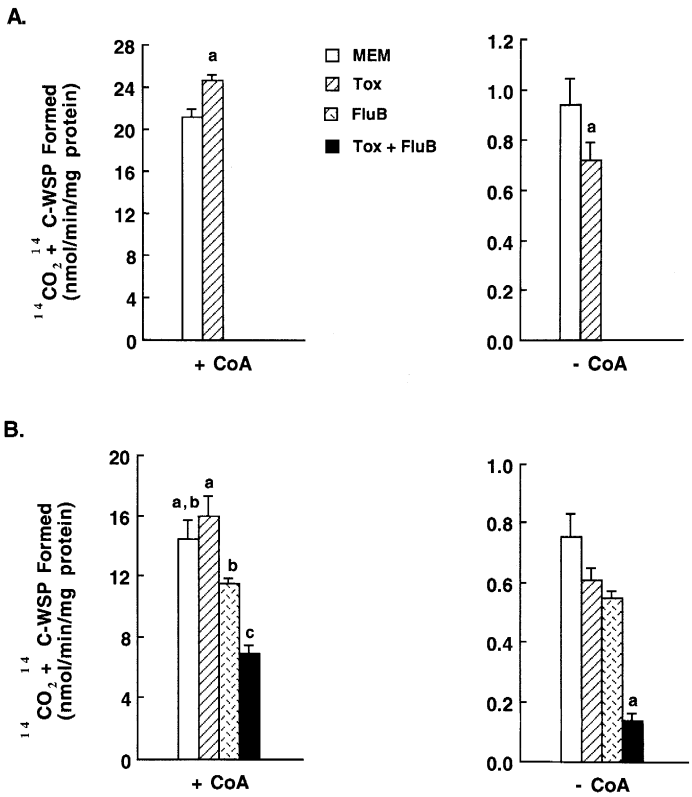

Fig. 1. Effects of exogenous CoA on hepatic [1-^{14}C]palmitic acid β-oxidation in vitro in the surfactant/virus mouse model for acute hepatic encephalopathy. A. Neonatal mice were painted on their abdomens daily for 12 days with either MEM or Toximul MP8 (Tox). On the final day of painting they were killed, and livers were homogenized immediately in 5 vol of Krebs-Henseleit buffer. The homogenates were then assayed for [1-^{14}C]palmitic acid β-oxidation in parallel, with or without exogenous CoA, as described in Section 2. B. After painting was discontinued, half of each of the MEM and Tox groups were inoculated with a sublethal dose (LD_{30}) of mouse-adapted human influenza B (Lee) virus (FluB); the remainder were given light anaesthesia only. 5 days after inoculation, livers from mice in each of the four treatment groups were assayed for [1-^{14} C]palmitic acid β-oxidation, with parallel assays running with or without exogenous CoA. The data are the mean ± SD of values obtained with 3–6 mice. Significant differences ($P < 0.05$) are indicated by different letters above the data bars.

theless, the system was sufficiently sensitive to detect the effects of the different treatment regimens in the mice. The day 12 livers demonstrated that an apparent stimulation of [1-^{14}C]palmitate oxidation by Tox painting was reversed with removal of exogenous CoA from the assay tubes (Fig. 1A). On day 19 (Fig. 1B), a Tox-induced inhibition of palmitate oxidation was still evident in the absense of added CoA. Of note was the observation that FluB infection resulted in an ~20% inhibition of palmitate oxidation, regardless of whether CoA was added to the assay. However, in its absence, the potentiation of the FluB effect by Tox (>80% inhibition, relative to MEM controls) was greater than the sum of the inhibitory effects of Tox (19%) or FluB (27%) alone.

These experiments were repeated using [1-^{14}C]octanoic acid as the substrate, since circulating levels of this C_8 fatty acid in serum of children with RS suggested that this could be the point during fatty-acid β-oxidation at which the metabolic block would be discriminatory [25]. The first observation was that oxidation of octanoate was much less dependent on exogenous CoA than was that of palmitate, as evidenced by the fact that oxidation rates were reduced by only about 45% (as opposed to 95% with palmitate) when CoA was omitted from the in vitro assays (Fig. 2). However, Tox-dependent decreases in octanoate oxidation at days 12 (Fig. 2A) and 19 (Fig. 2B) only occurred in the absence of exogenous CoA. As was the case with palmitate, FluB infection alone inhibited octanoate oxidation by ~30%, in a CoA-independent manner. Once again, Tox potentiation of FluB-induced inhibition of octanoate oxidation was most obvious when assays were carried out without exogenous CoA; in this case, the inhibition in the Tox + FluB group (>75%) was greater than the sum of that with livers from animals treated with Tox (8%) and FluB (30%) alone.

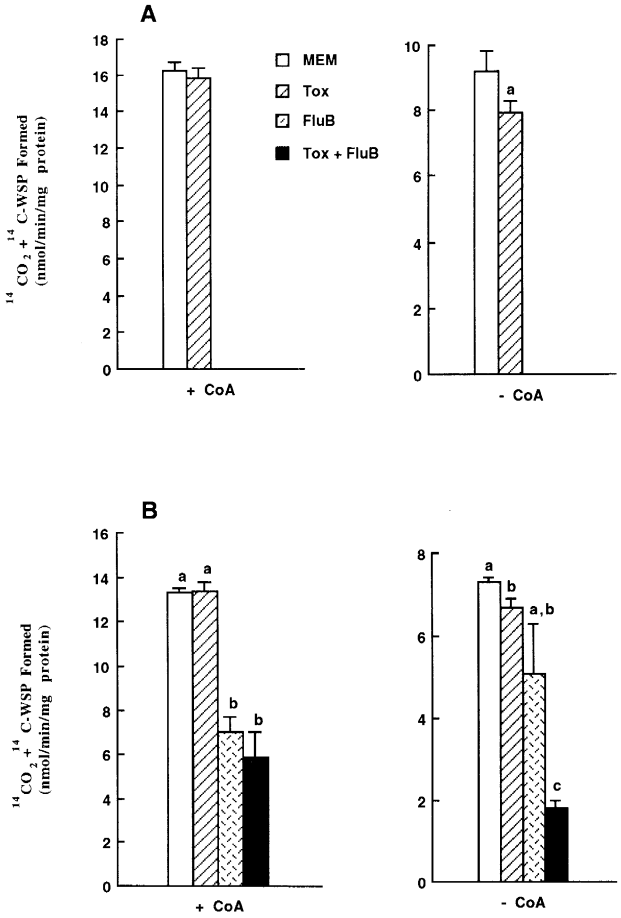

Fig. 2. Effects of exogenous CoA on hepatic [1-^{14}C]octanoic acid β-oxidation in vitro in the surfactant/virus mouse model for acute hepatic encephalopathy. A. Octanoate β-oxidation at day 12 in MEM- and Tox-treated mice, with or without exogenous CoA in the in vitro assay. B. Octanoate β-oxidation at day 19 in MEM-, Tox-, FluB- and Tox + FluB-treated mice, with or without exogenous CoA in the in vitro assay. Experimental details are in the legend for Fig. 1, or in Section 2. Data are the means ± SD of values obtained with 3–6 mice. Significant differences ($P < 0.05$) are indicated by different letters above the data bars.

3.3. Effects of Tox and FluB on hepatic CoA, acetyl-CoA and fatty acyl-CoA levels

Fig. 3A demonstrates that neither exposure of the mice to Tox for 12 days, their infection with FluB, nor the combined treatment with Tox + FluB altered hepatic total CoA levels. However, quantitation of the amounts of CoASH in the livers indicated that mice that had been painted with Tox for either 8 (not shown) or 12 days had levels of free CoA that were 20% lower than those of age-matched controls (Fig. 3B). This Tox-dependent decrease persisted through day 19 of the experiment, 6 days after cessation of painting; a similar magnitude of reduction in CoASH was seen in Tox + FluB-treated mice. This latter observation was consistent with the fact that FluB alone did not alter hepatic CoASH levels.

Although hepatic levels of total acid-soluble CoA esters were unaffected by Tox, FluB or Tox + FluB

treatments (not shown), there were significant effects on levels of acetyl-CoA (Fig. 3C). As was seen for CoASH, hepatic acetyl-CoA content was significantly depressed after the mice had been painted for 12 days with Tox; however, in the Tox alone group, this effect was reversed by day 19. Interestingly, FluB treatment alone significantly reduced acetyl-CoA relative to either MEM or Tox controls. A similar degree of reduction was also evident in the Tox + FluB group.

The only increases that were observed in hepatic CoA were those of the acid-insoluble acyl-CoA esters (Fig. 3D). These are usually esters of long-chain fatty acids such as palmitoyl-CoA, and could possibly include insoluble esters of Tox metabolites. Tox and FluB treatments independently resulted in significant increases in levels of this/these metabolites relative to age-matched controls. There was neither summation nor potentiation of this effect in the group given Tox + FluB.

3.4. Effects of Tox / FluB on hepatic [1-^{14}C]fatty acid:CoA ligase activities

Since Tox- and/or FluB-induced changes in hepatic CoA could affect fatty-acid activation we determined the activities of fatty acid:CoA ligase with [1-^{14}C]palmitic (Fig. 4A) and [1-^{14}C]octanoic (Fig. 4B) acids with the mouse livers. None of the experimental treatments affected [1-^{14}C]fatty acyl-CoA for-

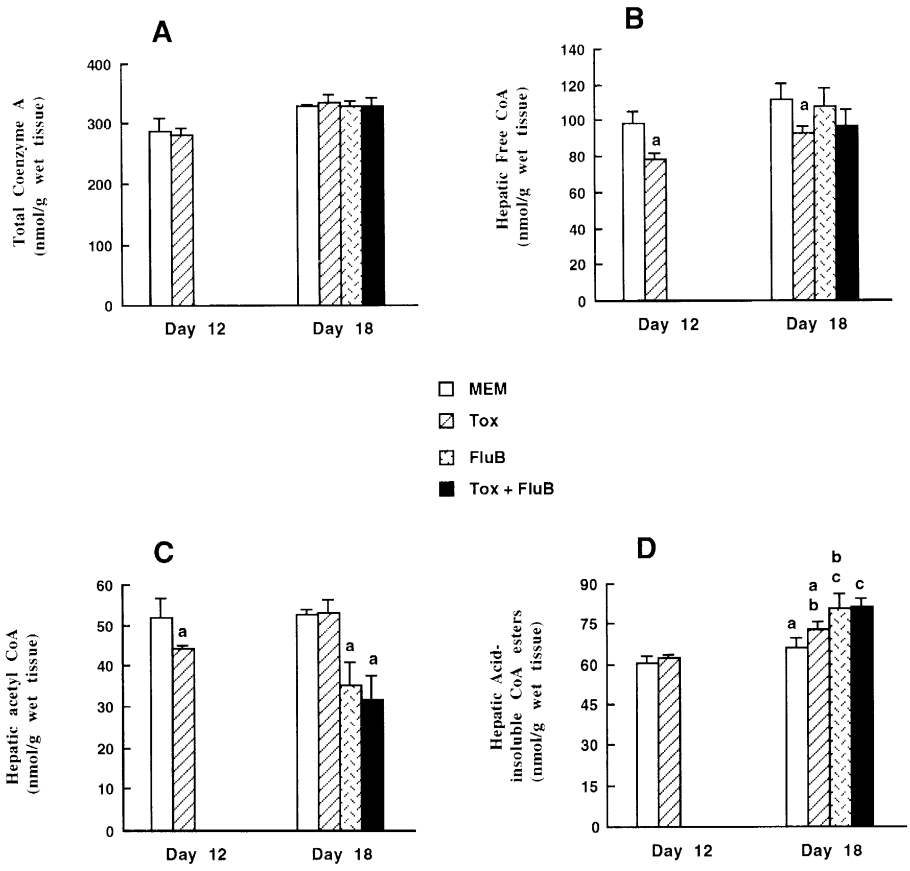

Fig. 3. Alterations in hepatic levels of total CoA (A), free CoA (B), acetyl-CoA (C) and acid-insoluble CoA esters (D) in the mouse model for AHE. Neonatal mice were painted on their abdomens daily for 12 days with either MEM or Toximul MP8 (Tox). On the final day of painting, 3–6 mice from each group were killed, and livers were frozen at −70°C. Half of each of the remaining groups were inoculated with a sublethal dose (LD_{30}) of mouse-adapted human influenza B (Lee) virus (FluB) and the remainder were given light anaesthesia only. Five days after inoculation, livers were taken and frozen. All samples were analyzed for free CoA and its derivatives using the enzyme cycling assay of McDougal and Dargar [23]. Data are the means ± SD of values obtained from 3–6 mice.

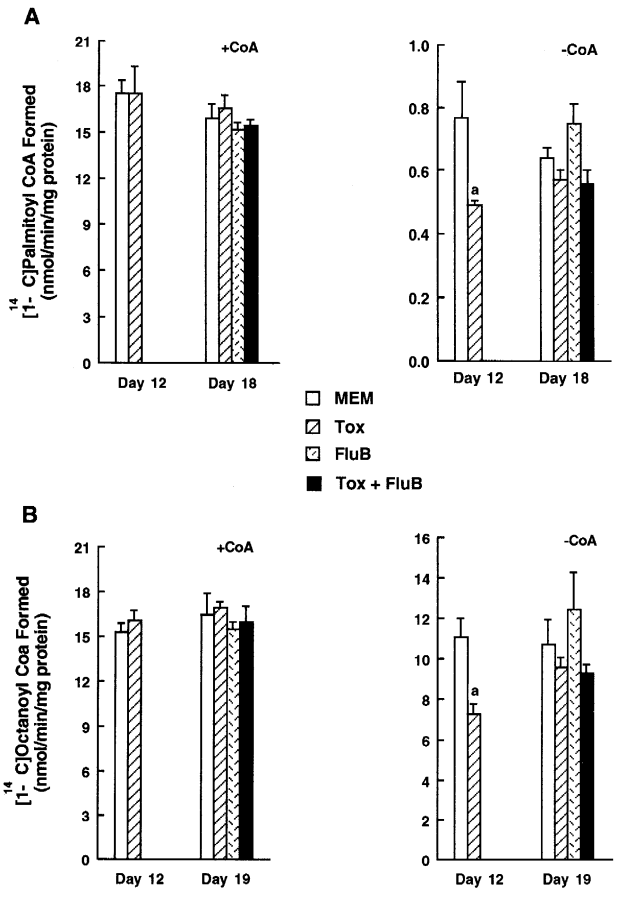

Fig. 4. Effects of Tox, FluB, and Tox + FluB treatment on activation of [1-^{14}C]palmitic (A) and [1-^{14}C]octanoic (B) acids. Mice were carried through the Tox/FluB model as described in Section 2, and in the legend to Fig. 1. The activities of fatty acid:CoA ligase were determined essentially as described by Singh and Poulos [24], in the absence or presence of exogenous CoA, as indicated. Data are the mean ± SD of values obtained from 3–6 mouse livers.

mation when exogenous CoA was included in the assay media. Several features were apparent when CoA was omitted. First, the activation rates in livers of control mice were significantly lower with palmitate than with octanoate. At day 12, activation of both fatty acids was significantly reduced in Tox-painted mice compared to controls (MEM), and the extent of inhibition ($\sim$ 35%) was similar for both fatty acids. By day 19, this effect appeared to be reversing, as the $\sim$ 10% inhibitions were not significantly different from values with the MEM controls or FluB-treated mice. Fatty acyl-CoA formation was not affected by FluB infection alone.

4. Discussion

This is the first report that β-oxidation is inhibited, and CoA metabolism is interrupted by dermal exposure to a widely-used industrial surfactant that in the past was considered to be a relatively inocuous compound. Most of the xenobiotics that have been recently shown to alter one or both events do so following ingestion of the agent [1]. Tox or one or more of its derivatives penetrate the skin, as measurable quantities were recovered from livers of humans that had been exposed to emulsifier-suspended pesticide and in livers of our Tox-treated mice [26]. Another unique feature of the study was that it demonstrated potentially important effects on intermediary metabolism of treatment with Tox or FluB. With few exceptions [27,28], studies have not investigated the combined effects of xenobiotics and viral or bacterial infections. Our mouse model involves both, since it has been used to mimic human RS, which is very often ($\geq$ 85%) associated with a viral infection [29].

Based on unusual hepatic acyl-CoA profiles, Corkey et al. proposed that several mitochondrial pathways, including fatty-acid β-oxidation, were impaired in RS patients [30]. Our studies demonstrated direct evidence that β-oxidation of both palmitic (Fig. 1) and octanoic (Fig. 2) acids is inhibited by either dermally exposing young mice to Tox or infecting them with FluB. Exogenous CoA could not be present in the in vitro assay to demonstrate the former; however, potentiation of FluB-induced inhibition was independent of its presence. Based on these observations, it was reasonable to assume that the Tox-mediated changes in fatty-acid oxidation that we reported recently [14] could involve effects on CoA metabolism, since many xenobiotic hydrocarbons are esterified with CoA in vivo [31]. The major components of Tox are dodecylbenzene sulfonates and nonylphenol ethoxylates of polyethylene glycol, which are anionic and nonionic surfactants, respectively. The former exist as branched chain (11–13 carbon atoms) or linear (8–9 carbon atoms) hydrocarbons that can undergo oxidative degration in vivo to the sulfonyl carboxylic acids that could be substrates for the ligase reaction [14,19,20]. The recent studies of Freneux et al. suggested that another xenobiotic, the 2-arylpropionate, ibuprofen, also altered hepatic

CoA metabolism [17]. These authors observed that reducing the amount of CoA in their incubations from 50 to 2.5 μM resulted in a reversal of the drug-dependent effect from stimulation (20%) to inhibition (45%) of palmitate β-oxidation. As in this study (Fig. 2), they found that β-oxidation of [1-^{14}C]octanoic acid was much less sensitive to changes in CoA availability, even in the presence of their inhibitory agent. It was interesting to note that their rates of β-oxidation with both fatty acids were considerably lower than those observed in this study, but this may be due to the fact that they used adult mice.

The hypothesis that Tox exposure altered CoA metabolism in the mouse model was supported by the data obtained in quantitative analyses of hepatic CoA species (Fig. 3). After 12 days of dermal painting, levels of free CoA (Fig. 3B) and acetyl-CoA (Fig. 3C) were significantly reduced relative to the MEM controls, although those of total hepatic CoA were unchanged (Fig. 3A). This finding was different from that in the rat aspirin + endotoxin model of Kilpatrick et al. [27], as in the latter, CoA and acetyl-CoA were unaffected by aspirin treatment alone. In another model, injection of suckling mice with valproic acid reduced hepatic levels of both free CoA and acetyl-CoA [32]; however, acid-soluble short- and medium-chain acyl-CoA and total CoA levels were also elevated significantly, and this was not seen in Tox-treated mice. Kang et al. reported that levels of these metabolites were also elevated in autopsy liver samples of children who had died of RS [33]. These inconsistencies in the effects of different xenobiotics and other contributing factors on CoA metabolism may reflect different mechanisms of action, and/or different target sites within the cell.

In addition to its effects on CoA metabolism, it was also possible that Tox metabolites in vivo could influence the activity of fatty-acyl CoA ligase, the enzyme(s) responsible for activating fatty acids to their corresponding CoA esters. This was confirmed by analysis of the enzyme activity with [1-^{14}C]palmitic and [1-^{14}C]octanoic acids (Fig. 4). Of particular interest was the observation that Tox-dependent inhibition of ligase activity was only evident when assays were carried out in the absence of CoA. This could explain the recently-reported absence of effect on ligase activity of many β-oxidation-inhibiting agents [34], since these investigators carried out their assays in the presence of relatively high (0.6 mM) quantities of CoA. Whether the inhibitions observed in this model were due to direct Tox-dependent inhibition of enzyme activity, or to competition for it by Tox, is not known at present. The difference between the levels of ligase activity with palmitate and octanoate that were obtained when the assays were carried out in the absence of exogenous CoA could be explained by the fact that they were done using liver homogenates. Depletion of extramitochondrial CoA by Tox would reduce the activation of palmitic acid selectively, as this reaction occurs at the outer mitochondrial membrane. By contrast, octanoate activation does not depend upon cytosolic CoA, as it can cross directly into the mitochondria where medium-chain acid CoA ligase and an independent, concentrated supply of CoA are available.

As discussed previously [14], the mechanism(s) whereby FluB, a respiratory virus, inhibits β-oxidaton is not known. Isolation of active virus from the mouse livers has not been demonstrated, however, we have evidence that with pre-exposure to Tox, non-infective FluB genomic material does invade this normally non-target organ (unpublished observations). An alternative explanation which could account for inhibition of fatty acid oxidation in mice that were only infected with FluB is that the virus could produce metabolites, including cytokines [35], that could inhibit enzyme activities, either directly or through reduced enzyme synthesis, at targets distant from the site of infection. Presumably the 27–30% inhibition of palmitate (Fig. 1) and octanoate (Fig. 2) oxidation in mice inoculated with FluB alone is directly associated with the 33% reduction in acetyl-CoA levels (Fig. 3C) and the approximate 22% increase in acid-insoluble acyl-CoA esters (Fig. 3D) in this model.

The only biochemical correlate of Tox potentiation of FluB-induced mortality in our mouse model is the potentiation by Tox of hepatic FluB-dependent inhibition of β-oxidation (Fig. 1B, Fig. 2B). This investigation of the effects of the different treatment regimens on levels of hepatic CoA and its metabolites and of the activity of fatty acyl-CoA ligase did not reveal any other specific aspects of cell metabolism in which abnormalities were potentiated when mice were given combined treatment with Tox and FluB. Each of these insults alone has significant effects on cellular metabolism; however, the precise basis for

the synergism seen in inhibition of β-oxidation and mortality remains unidentified.

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References

[1] B. Fromenty, D. Pessayre, Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity, Pharmacol. Ther. 67 (1995) 101–154.
[2] M. Visentin, M. Salmona, M.T. Tacconi, Reye's and Reye-like syndromes, drug-related diseases? (causative agents, etiology, pathogenesis, and therapeutic approaches). Drug Metab. Rev. 27, 1995, 517–539.
[3] D. Deschamps, C. Fisch, B. Fromenty, A. Berson, C. Degott, D. Pessayre, Inhibition by salicylic acid of the activation and thus oxidation of long chain fatty acids. Possible role in the development of Reye's Syndrome, J. Pharmacol. Exp. Ther. 259 (1991) 894–904.
[4] Y. Yoshida, M. Fujii, F.R. Brown, I. Singh, Effects of salicylic acid on mitochondrial-peroxisomal fatty acid catabolism, Pediatr. Res. 23 (1988) 338–341.
[5] G. Labbe, B. Fromenty, E. Freneaux, V. Morxelle, P. Lerreron, A. Berson, D. Pessayre, Effects of various tetracycline derivatives on in vivo and in vitro β-oxidation of fatty acids, egress of triglycerides from the liver, accumulation of hepatic triglycerides, and mortality in mice, Biochem. Pharmacol. 41 (1991) 638–641.
[6] P.B. Mortensen, Inhibition of fatty acid oxidation by valproate, Lancet 2 (1980) 856–857.
[7] M. Yamamoto, Y. Nakamura, Inhibition of β-oxidation by 3-mercaptopropionic acid produces features of Reye's Syndrome in perfused rat liver, Gastroenterology 107 (1994) 517–524.
[8] K. Tanaka, Y. Ikeda, Hypoglycin and Jamaican vomiting sickness, Prog. Clin. Biol. Res. 321 (1990) 167–184.
[9] J.F.S. Crocker, K.R. Rozee, R.L. Ozere, S.C. Digout, O. Hutzinger, Insecticide and viral interactions as a cause of fatty visceral changes and encephalopathy in the mouse, Lancet 2 (1974) 22–24.
[10] J.F.S. Crocker, R.L. Ozere, S.H. Safe, S.C. Digout, K.R. Rozee, O. Hutzinger, Lethal interactions of ubiquitous insecticide carriers with virus, Science 192 (1976) 1351–1354.
[11] J.F.S. Crocker, K.W. Renton, S.H. Lee, K.R. Rozee, S.C. Digout, D.A. Malatjalian, Biochemical and morphological characteristics of a mouse model of Reye's Syndrome induced by the interaction of influenza B virus and a chemical emulsifier, Lab. Invest. 54 (1986) 32–40.
[12] J.F.S. Crocker, S.H.S. Lee, J.A. Love, D.A. Malatjalian, R.W. Renton, K.R. Rozee, M.G. Murphy, Surfactant-potentiated increases in intracranial pressure in a mouse model of Reye's Syndrome, Exp. Neurol. 111 (1991) 95–97.
[13] M.G. Murphy, L. Archambault-Schertzer, J. Van Kessel, S.C. Digout, D.A. Malatjalian, J.F.S. Crocker, Hepatic lipid abnormalities in a chemical/viral mouse model for Reye's Syndrome, Lipids 22 (1987) 217–223.
[14] M.G. Murphy, J.F.S. Crocker, H. Her, Abnormalities in hepatic fatty-acid metabolism in a surfactant/influenza B virus mouse model for acute encephalopathy, Biochim. Biophys. Acta 1315 (1996) 208–216.
[15] J.H. Thurston, R.E. Hauhart, A single therapeutic dose of valproate affects liver carbohydrate, fat, adenylate, amino acid, coenzyme A, and carnitine metabolism in infant mice: possible clinical significance, Life Sci. 36 (1985) 1643–1651.
[16] S. Ponchaut, F. Van Hoof, K. Veitch, In vitro effects of valproate and valproate metabolites on mitochondrial oxidations. Relevance of CoA sequestration on the observed inhibitions, Biochem. Pharmacol. 43 (1993) 2435–2442.
[17] E. Freneaux, B. Fromenty, A. Berson, G. Labbe, C. Degott, P. Letteron, D. Larrey, D. Pessayre, Stereoselective and nonstereoselective effects of ibuprofen enantiomers on mitochondrial β-oxidation of fatty acids, J. Pharmacol. Exp. Ther. 255 (1990) 529–535.
[18] L.J. Ruff, E.P. Brass, Metabolic effects of pivalate in isolated rat hepatocytes, Toxicol. Appl. Pharmacol. 110 (1991) 295–302.
[19] S. Safe, O. Hutzinger, J.F.S. Crocker, The role of chemicals in Reye's Syndrome. in: J.F.S. Crocker (Ed.), Reye's Syndrome II, Grune and Stratton, New York, 1979, pp. 281-309.
[20] J.G. Black, D. Howes, D. Absorption, metabolism, and excretion of anionic surfactants. in: G. Gloxhuber, K. Kunstler (Eds.), Anionic Surfactants, Marcel Dekker Inc., New York, 1992, pp. 43-79.
[21] O.H. Lowry, H.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[22] B. Fromenty, E. Freneaux, G. Labbe, D. Deschamps, D. Larrey, P. Letteron, D. Pessayre, Tianeptine, a new tricyclic antidepressant metabolized by β-oxidation of its heptanoic side chain, inhibits the mitochondrial oxidation of medium and short chain fatty acids in mice, Biochem. Pharmacol. 38 (1989) 3743–3751.
[23] D.B. McDougal Jr., R.V. Dargar, A spectrophotometric cycling assay for reduced coenzyme A and its esters in small amounts of tissue, Analyt. Biochem. 97 (1979) 103–115.
[24] H. Singh, A. Poulos, Distinct long chain and very long chain fatty acyl CoA synthetases in at liver peroxisomes and microsomes, Arch. Biochem. Biophys. 266 (1988) 486–495.
[25] W.D. Parker Jr., R. Haas, D.A. Stumpf, L.A. Eguren, Neurology 33 (1983) 1374–1377.

[26] J.F.S. Crocker, D. Fung, R. Hudson, S.H. Safe, Examination of the role of surfactants in Reye's Syndrome. in Reye's Syndrome IV, J.D. Pollack (Ed.), The National Reye's Syndrome Foundation, Bryan, Ohio, 1985, pp. 135-140.

[27] L.E. Kilpatrick, R.A. Polin, S.D. Douglas, B.E. Corkey, Hepatic metabolic alterations in rats treated with low-dose endotoxin and aspirin: An animal model of Reye's Syndrome, Metabolism 38 (1989) 73–77.

[28] D.A. Trauner, E. Horvath, L.E. Davis, Inhibition of fatty acid beta oxidation by influenza B virus and salicylic acid in mice: Implications for Reye's syndrome, Neurology 38 (1988) 239–241.

[29] C.C. Linnemann, Jr., L. Shea, C.A. Kauffman and G.M. Schiff, Association of Reye's Syndrome with viral infection. Lancet 2, 1974, 179-182.

[30] B.E. Corkey, D.E. Hale, M.C. Glennon, R.I. Kelley, P.M. Coates, L. Kilpatrick, C.A. Stanley, Relationship between unusual hepatic acyl coenzyme A profiles and the pathogenesis of Reye Syndrome, J. Clin. Invest. 82 (1988) 782–788.

[31] P. Dodds, Xenobiotic lipids: The inclusion of xenobiotic compounds in pathways of lipid biosynthesis, Prog. Lipid Res. 34 (1995) 219–247.

[32] J.H. Thurston, R.E. Hauhart, Reversal of the adverse chronic effects of the unsaturated derivative of valproic acid — 2-*n*-propyl-4-pentenoic acid — on ketogenesis and liver coenzyme A metabolism by a single injection of pantothenate, carnitine, and acetylcysteine in developing mice, Pediatr. Res. 33 (1993) 72–76.

[33] E.S. Kang, M.T. Capaci, D.N. Korones, N. Tekade, Liver coenzyme A ester content: comparison between Reye's syndrome and control subjects, Clin. Sci. 63 (1982) 455–460.

[34] B.J. Roberts, K.M. Knights, Inhibition of rat peroxisomal palmitoyl-CoA ligase by xenobiotic carboxylic acids, Biochem. Pharmacol. 44 (1992) 261–267.

[35] N. Iyngkaran, M. Yadav, F. Harun, K.R. Kamath, Augmented tumour necrosis factor in Reye's Syndrome associated with dengue virus, Lancet 340 (1992) 1466–1467.