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Toxic Effects of Fatty Acid Anilides on the Oxygen Defense Systems of Guinea Pig Lungs and Erythrocytes

Shyamali Mukherjee, Sukla Ghosh, Lisa Rodgers, Tultul Nayyar, Usha Desai, and Salil K. Das

Department of Biochemistry, Meharry Medical College, Nashville, TN 37208

ABSTRACT: Toxic oil syndrome (TOS) is caused by ingestion of denatured edible oils. Even though the etiology and pathogenesis of this disease are not fully known, it is quite clear that generation of free radicals caused by ingestion of fatty acid anilides is responsible for the pathogenetic mechanism in many TOS patients. Fatty acid anilides may also alter the free radical status of lungs and erythrocytes; this possibility may shed some light on understanding toxic oil syndrome. The present study describes the effects of oral administration of fatty acid anilides on the activities of major enzymes involved in the oxygen defense systems of lungs and erythrocytes. Feeding fatty acid anilides caused an increase in the superoxide dismutase (SOD) activity in erythrocytes, whereas it caused a decrease in the SOD activity in lungs. GSH-Px activity was not significantly changed in erythrocytes but was decreased in lungs. Although the activity of catalase was increased only by a higher dose in the erythrocytes, it was not affected in the lung at any dosage. Even though the ingestion of fatty acid anilides caused an increase in the SOD activity in the erythrocytes and a decrease in the SOD activity in the lungs, there was an increase in the lipid peroxidation in both cases. The increase in lipid peroxidation in erythrocytes is probably caused by the accumulation of H_2O_2, and that in the lungs is due to the accumulation of superoxide anion.

KEYWORDS: **Lung, Oleylanilide, Linoleylanilide, Oxygen Defense Systems, Lipid Peroxidation, Guinea Pigs.**

INTRODUCTION

Toxic oil syndrome (TOS), a condition first reported in Spain in 1981, is a multisystemic disorder caused by consumption of edible oils denatured with aniline (1–3). The symptoms of the disease in its acute stage are respiratory distress, fever, headache, itching, nausea, and sometimes muscular pains and neurological disorders. Lung damage has been responsible for most of the early TOS deaths. Even though the etiology and pathogenesis of this disease are not fully known, it is clear that respiratory complications leading to pulmonary hypertension, pulmonary edema, hydropic degeneration of type I and type II pneumocytes and adult respiratory distress syndrome (ARDS) do arise in many TOS patients (4,5). Unfortunately, however, the exact mechanism through which pulmonary complications in TOS patients arise has not yet been established. It has been suggested that the pathology of this disease may be related to the peroxidation of membrane lipids induced by one or more of the toxins present in the industrial oils (6).

It should be noted that generation of free radicals caused by fatty acid anilides present in the denatured oils have been thought to be responsible for the pathogenetic mechanism in the lungs of TOS patients (7–11). These reports suggest that free radicals generated from denatured oils may produce endothelial lesions that could lead to proliferation and vascular fibrosis commonly seen in toxic-allergic syndrome (4,10). Lung injury may also result from intracellular oxygen-derived free radical over production (12) or from toxic products released by phagocytes, such as polymorphonuclear neutrophils (13). Under these circumstances, oxygen metabolite production exceeds the capacity of the cellular antioxidant defense mechanisms (14). The sensitivity of specific cell types to oxygen injury could be related in part to the activities of endogenous cellular antioxidant enzymes. Other plausible mechanisms for tissue damage are the free

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Address correspondence to Salil K. Das, Department of Biochemistry, Meharry Medical College, Nashville, TN 37208.

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radical-mediated peroxidation of lipids and proteins and the numerous reactive degradation products. These entities facilitate intramolecular and intermolecular crosslinking among lipids and proteins (15).

Research directed toward a better understanding of TOS has been limited because of both the practical and ethical difficulties in working with humans and the discrepancies that result in the analysis of human data. It is thus imperative for an appropriate animal model to be developed to study the pathogenetic determinants of TOS and to explore possible therapeutic intervention. Use of this animal model to investigate the effects of anilide consumption on the oxygen defense systems of both the lungs and the red blood cells could prove consequential in the further study of the postulated relationship between oxygen consumption and oxygen transport in the ARDS induced by toxic oil ingestion (16). The present study focuses on the effects of the ingestion of two fatty acid anilides, oleylanilide (OA) and linoleylanilide (LA), on the oxygen defense systems of guinea pig lungs and erythrocytes. We reported earlier that in some metabolic characteristics, such as lung surfactant development, guinea pigs are more similar to human than are other animal species (17); hence guinea pigs are used as animal model.

MATERIALS AND METHODS

Fatty Acid Anilides

We used two samples of oleylanilide, one synthesized by us (OA-1) and the other (17–89–18A) provided by Angel Messeguer, Department of Biological Organic Chemistry, Centro de Investigacion Y Desarrollo, Barcelona, Spain. Linoleylanilide (22–89–20A) and two other compounds that present physical similarities with the anilides, L-tetradecanol (00–89–14A) and L-hexadecanol (00–89–16A), were also obtained from Dr. Messeguer. Tetradecanol and hexadecanol served as blanks for comparison.

Synthesis of OA

Equimolecular amounts of oleic acid, aniline, and dimethylaminopropylethyl carbodi-imide dissolved in ethanol were kept at room temperature for three days (1). The anilide was obtained after concentrating the solution. The crude product was recrystallized from ethanol and dried under vacuum. Mass spectrometric analysis confirmed the molecular weight of the synthetic compound to be 357.

Animals

Adult male guinea pigs, Hartley strain, weighing approximately 250 grams, were obtained from Camm Laboratory Animals (Wayne, NJ). All animals were housed in rooms with laminar flow of filtered air. Food and water were offered ad libitum. In phase 1 of the study, we investigated the effects of different concentrations of OA-1 on the oxygen defense status of erythrocytes and lungs. For this study, the animals were divided into 5 groups with 6 animals in each group. Group I served as the control and each control animal received 0.5 ml pure olive oil daily, delivered by an oesophageal tube. Animals from groups II, III, IV, and V received, by oesophageal tube daily, a suspension of OA-1 in olive oil at 35, 50, 100, and 150 mg per kg body weight, respectively. The experiment was conducted for 30 days, and the body weight gain of each animal was recorded.

In phase 2 of the study, we investigated the anilide compounds received from Barcelona, Spain. For this study, the animals were divided into five groups with six animals in each group. Group I served as the control and received the vehicle only. Group II and group III served as blanks for comparison and received daily 150 mg/kg of L-tetradecanol (00–89–14A) and L-hexadecanol (00–89–16A), respectively. Group IV and group V received daily 150 mg/kg of 17–89–18A and 22–89–20A, respectively. This experiment was continued for 45 days, and the body weight gain of each animal recorded.

At the end of the experiments, the guinea pigs were anesthetized with sodium pentobarbital containing heparin (10 mg pentobarbital and 400 units heparin/100 gm body weight). Approximately 5 ml of blood were collected by cardiac puncture for biochemical analysis. The chest cavity was opened and a thoracotomy was done. Following the thoracotomy, the pulmonary vasculature was lavaged by sterile normal saline injected through the right ventricle. The lungs were then removed for biochemical analysis.

Analysis of Oxygen Defense Mechanisms of Erythrocytes and Lungs

Erythrocytes were isolated from the blood as described earlier (18). Aliquots of the erythrocyte suspension were taken for the counting of cells with a Coulter Counter ZBI (19). Lungs were homoge-

nized in a buffer containing 0.25 M sucrose, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and aprotinin (2 μg/ml). They were centrifuged at 200 × g for 10 minutes to discard the unbroken tissue, and the supernatant was collected to determine the oxygen defense status.

The activity of superoxide dismutase (SOD) was measured by the procedure of Hyland *et al.* (20). Glutathione peroxidase (GSH-Px) activity was measured by the method of Flohe and Gunzler (21). Catalase activity was determined according to the method of Sinha (22). The activity of glucose 6-phosphate dehydrogenase (G6PD) and that of 6-phosphogluconate dehydrogenase (6PGD) was measured by the method of Beutler (23).

The susceptibility of erythrocyte and lung tissue lipid to auto-oxidation (peroxidation potential) was assessed by the measurement of malonaldehyde (MDA)-thiobarbituric acid (TBA) derivative with absorbance at 532 nm using the method of Stocks & Dormandy (24). However, because Gilbert *et al.* (25) reported that erythrocyte peroxidation produces TBA reactive substances other than MDA, it is necessary to correct for the interference results. Gilbert *et al.* (25) demonstrated and later we confirmed (26) that 20% of the intensity of the maximum absorption peak at 453 nm represents the TBA reactive substances other than MDA. Therefore, the correct MDA values were obtained by subtracting 20% of the absorbance at 453 nm from the absorbance at 532 nm. In some experiments, erythrocytes were treated with a 5 mM solution of sodium azide to inhibit catalase activity irreversibly (27). The protein content of the erythrocyte suspension and lung homogenate was measured by the method of Peterson (28).

Statistical Analysis

Data were treated statistically using the Student's t test (29). The variability of data is presented as mean ± standard error. Differences at $p < 0.05$ were considered significant.

RESULTS

Body Weight Gain

Table 1 presents data on the effects of fatty acid anilides ingestion on the body weight gain of the animals. In comparison to the control group receiving olive oil, all other groups that received either the alcohols (tetradecanol and hexadecanol) or the two fatty acid anilides had less body weight gain. There was no difference between the animals receiving the alcohols and those receiving the fatty acid anilides. As the dose of oleylanilide increased, there was a corresponding decrease in the body weight gain.

TABLE 1. Effects of Fatty Acid Anilides on Body Weight Gain of Guinea Pigs

Animals	*At Start gram*	*At End gram*	*Gain gram*
Phase 1			
Control	245.5 ± 1.7	584.7 ± 4.3	339.2
Experimental (OA-1)			
35 mg/kg	183.2 ± 3.4	452.3 ± 6.9	269.1*
50 mg/kg	229.1 ± 0.7	484.4 ± 2.8	255.3*
100 mg/kg	264.1 ± 0.4	572.9 ± 2.9	308.8*
150 mg/kg	261.4 ± 1.8	453.5 ± 4.2	192.1*
Phase 2			
Control	217.0 ± 2.0	510.5 ± 5.0	293.5
Experimental (150 mg/kg)			
00–89–14A	269.5 ± 8.3	450.3 ± 12.3	180.8*
00–89–16A	262.8 ± 2.9	490.7 ± 9.8	227.5*
17–89–18A	281.0 ± 8.2	505.2 ± 19.3	224.2*
22–89–20A	270.8 ± 5.7	490.5 ± 10.0	219.2*

Values are mean ± SE of six animals. *Significantly different from control. OA-1 = oleylanilide synthesized by us; 00–89–14A = L-tetradecanol; 00–89–16A = L-hexadecanol; 17–89–18A = oleylanilide obtained from Spain; 22–89–20A = linoleylanilide obtained from Spain; control = olive oil. 00–89–14A and 00–89–16A served as blanks for comparison.

SOD, GSH-Px, and Catalase Activities of Erythrocytes

As shown in Table 2, the activity of SOD was increased significantly at all doses of OA-1, and the increase was dependent on the dose used. There was no significant change in the activity of GSH-Px; however, the activity of catalase was increased only at high dose of OA-1. Similar data were obtained with the two fatty acid anilides obtained from Barcelona. However, there was no significant difference between the control group and those receiving the alcohols.

In Vitro Lipid Peroxidation of Erythrocytes

As shown in Table 3, there was a significant increase in the lipid peroxidation potential in erythrocytes ($p < 0.05$) caused by the ingestion of both anilides. There was no variation between the control group and those receiving the alcohols.

TABLE 2. Effects of Fatty Acid Anilides Ingestion on the Activities of SOD, GSH-Px and Catalase of Guinea Pig Erythrocytes

Animals	*SOD*	*GSH-Px*	*Catalase*
Phase 1			
Control	161.9 ± 6.7	0.7 ± 0.1	10.3 ± 1.7
Experimental (OA-1)			
35 mg/kg	199.4 ± 4.9*	1.0 ± 0.3	12.3 ± 1.9
50 mg/kg	241.7 ± 4.0*	0.9 ± 0.1	14.9 ± 2.9
100 mg/kg	287.2 ± 6.0*	1.3 ± 0.5	20.2 ± 2.5*
150 mg/kg	290.5 ± 5.2*	1.3 ± 0.6	22.0 ± 2.8*
Phase 2			
Control	157.0 ± 9.8	0.8 ± 0.1	9.9 ± 1.3
Experimental (150 mg/kg)			
00–89–14A	174.0 ± 5.2	0.9 ± 0.2	9.2 ± 1.3
00–89–16A	165.8 ± 18.6	0.9 ± 0.2	10.5 ± 0.1
17–89–18A	364.0 ± 15.4*	1.4 ± 0.4	29.9 ± 0.8*
22–89–20A	325.8 ± 8.0*	1.2 ± 0.1	24.1 ± 0.5*

Values are expressed as mean ± SE of 6 animals per cell. SOD activity is expressed as units × 10^{-9}, GSH-Px activity is expressed as umoles × 10^{-9} NADPH oxidized/min, catalase activity is expressed as umoles × 10^{-7} H_2O_2 decomposed/min. *Statistically significant difference from control.

TABLE 3. Effects of Fatty Acid Anilides Ingestion on the in Vitro Lipid Peroxidation of Erythrocytes

	Endogenous	*RBC treated with H_2O_2*	
Animals	*MDA*	*$-NaN_3$*	*$+NaN_3$*
Phase 1			
Control	12.6 ± 0.1	344.8 ± 6.0	657.0 ± 4.0
Experimental (OA-1)			
35 mg/kg	21.4 ± 0.3*	637.1 ± 5.0*	874.3 ± 9.6*
50 mg/kg	40.3 ± 0.2*	606.5 ± 5.0*	1087.4 ± 4.4*
100 mg/kg	50.6 ± 0.3*	778.5 ± 6.0*	1120.3 ± 5.0*
150 mg/kg	62.3 ± 0.4*	857.1 ± 2.0*	1230.2 ± 4.1*
Phase 2			
Control	11.3 ± 0.1	561.2 ± 1.0	843.0 ± 2.0
Experimental (150 mg/kg)			
00–89–14A	12.1 ± 0.1	460.5 ± 2.0	960.5 ± 5.0
00–89–16A	11.0 ± 0.1	421.3 ± 3.0	870.5 ± 8.0
17–89–18A	68.1 ± 0.5*	952.0 ± 1.7*	1500.3 ± 9.0*
22–89–20A	55.3 ± 0.8*	830.5 ± 4.0*	2460.5 ± 1.6*

Values are mean ± SE of 6 animals. Values are expressed as nmoles × 10^{-9} thiobarbituric reactive substances (MDA) formed/cell.
*Statistically different from control.

SOD, GSH-Px, and Catalase Activities of Lung

For the evaluation of the oxygen defense status in lung, we determined the activity of SOD, GSH-Px, catalase, G6PD, and 6PGD. Data are shown in Table 4. No remarkable change occurred in the activities of catalase and G6PD, however, the activities of SOD, GSH-Px, and 6PGD decreased significantly in the animals receiving the fatty acid anilides ($p < 0.05$). Again, there was no difference between the control group and those receiving the alcohols.

In Vitro Lipid Peroxidation of Lung

Data on the lipid peroxidation potential are shown in Table 5. There was a significant increase in lipid peroxidation potential in the lung of the fatty acid anilides treated groups. There was no difference between the control group and those receiving the alcohols.

DISCUSSION

Even though the development of TOS in humans has been attributed to the consumption of edible oils denatured with aniline, it is difficult to establish either the amount or the dose of fatty acid anilides consumed by the TOS patients. The present study strongly suggests that the degree of modulation of the oxygen defense system in guinea pig lungs and erythrocytes depends on the dose of oleylanilide consumed.

As compared to the control group, the experimental animals showed 9% to 44% depression in body weight gain over the 30 day period as the dose of oleylanilide was increased from 35 to 100 mg/kg body weight (phase 1, Table 1). It is interesting to note that animals receiving tetradecanol and hexadecanol also had a depression in body weight gain in comparison to the control group (phase 2, Table 1). We do not have any explanation for this observation.

As the dose of oleylanilide increased, there was a corresponding increase in the activity of SOD in erythrocytes. However, there was no corresponding increase in the combined activity of GSH-Px and catalase. Thus, there was an imbalance between the SOD activity and the activity of the two peroxide scavengers. This imbalance may have caused an excessive accumulation of hydrogen peroxide in erythrocytes leading to increased lipid peroxidation (Table 3).

Anilide ingestion has been reported to cause increased lipid peroxidation in membrane fraction of liver extracts of rats (30) and in tissues of TOS patients (31). The increased lipid peroxidation in erythrocytes caused by anilides ingestion as observed in our study may be associated with (1) an increased production of hydroxyl radicals by Fenton-type reactions with Cu and Fe, and (2) a redox cycling of free iron liberated from the unstable hemoglobin (32). Superoxide radicals have indeed been shown to be generated in the red blood cells when oxyhemoglobin is autoxidized to methemoglobin (33). It has also been reported that the methemoglobin level is higher in blood of TOS pa-

TABLE 4. Effects of Fatty Acid Anilides Ingestion on the Activities of SOD, GSH-Px, Catalase, G6PD and 6PGD of Guinea Pig Lung

Animals	*SOD*	*GSH-Px*	*Catalase*	*G6PD*	*6PGD*
Phase 1					
Control	609.4 ± 2.9	2.8 ± 0.1	30.2 ± 0.2	574.6 ± 6.2	670.3 ± 5.3
Experimental (OA-1)					
35 mg/kg	527.7 ± 9.0*	2.6 ± 0.1	34.9 ± 0.6	559.9 ± 3.7	608.3 ± 2.8
50 mg/kg	444.0 ± 8.3*	1.5 ± 0.0*	31.5 ± 0.9	512.3 ± 4.5	535.5 ± 4.5
100 mg/kg	392.7 ± 5.0*	1.8 ± 0.1*	32.7 ± 0.4	508.3 ± 6.2	400.2 ± 3.2
150 mg/kg	390.6 ± 5.6*	1.9 ± 0.1*	33.5 ± 0.3	500.5 ± 3.2	379.3 ± 6.5
Phase 2					
Control	619.4 ± 4.1	2.9 ± 0.1	31.2 ± 4.1	560.9 ± 7.1	690.3 ± 7.3
Experimental (150 mg/kg)					
00-89-14A	591.1 ± 9.8	2.5 ± 0.1	41.4 ± 0.3	611.6 ± 6.1	648.4 ± 7.2
00-89-16A	581.1 ± 6.0	2.2 ± 0.1	44.5 ± 0.4	554.2 ± 7.2	614.3 ± 8.2
17-89-18A	387.8 ± 9.7*	1.6 ± 0.1*	37.1 ± 0.2	507.9 ± 9.2	389.7 ± 6.1*
22-89-20A	419.6 ± 6.6*	1.8 ± 0.1*	30.5 ± 0.2	524.7 ± 8.2	348.6 ± 5.2*

Values are expressed as mean ± SE of 6 samples per gram tissue. SOD activity is expressed as units, GSH-Px activity is expressed as umoles NADPH oxidized/min, catalase activity is expressed as umoles H_2O_2 decomposed/min, G6PD and 6PGD activities are expressed as umoles NADP reduced/min. *Statistically significant difference from control.

TABLE 5. Effects of Fatty Acid Anilides Ingestion on in Vitro Lipid Peroxidation of Guinea Pig Lung

Animals	*Endogenous MDA*	*Lung treated with H_2O_2*	
		$-NaN_3$	*$+NaN_3$*
Phase 1			
Control	69.5 ± 2.0	145.3 ± 7.2	287.8 ± 2.5
Experimental (OA-1)			
35 mg/kg	75.1 ± 3.1	194.5 ± 4.0	373.7 ± 6.4*
50 mg/kg	81.8 ± 0.7	212.3 ± 5.5	442.4 ± 4.4*
100 mg/kg	89.4 ± 1.4	221.4 ± 3.9	495.5 ± 3.2*
150 mg/kg	97.5 ± 3.2	260.5 ± 1.6	558.2 ± 4.1*
Phase 2			
Control	71.6 ± 6.1	140.5 ± 6.1	267.8 ± 21.6
Experimental (150 mg/kg)			
00-89-14A	75.3 ± 5.1	113.8 ± 2.7	248.0 ± 21.5
00-89-16A	75.0 ± 7.2	189.2 ± 18.8	337.2 ± 13.3
17-89-18A	107.5 ± 5.4	273.4 ± 18.1	649.8 ± 28.8*
22-89-20A	83.9 ± 13.8	331.0 ± 43.8	538.3 ± 28.0*

Values are mean ± SE of 6 animals. Values are expressed as nmoles thiobarbituric reactive substances (MDA) formed per mg lung protein. *Statistically different from control.

tients (34). It is also known that superoxide radicals can accelerate the metal ion-dependent formation of highly reactive hydroxyl radical from H_2O_2 (35). However, we have not yet studied whether there is a perturbation of iron metabolism due to anilides ingestion.

Fatty acid anilides ingestion caused a significant decrease in the activities of SOD and GSH-Px without any effect on the activity of catalase in the lung (Table 4). This suggests that there will be less dismutation of superoxide radicals generated in the lung of animals treated with anilides and as a result there will be accumulation of superoxide anion.

It is known that superoxide radicals can inhibit catalase (36) and glutathione peroxidase (37), and H_2O_2 can inhibit SOD (38). It has been reported that the inhibition of SOD in rat lungs can lead to the inactivation of GSH-Px (39), which further illustrates the interdependence of these enzymes. SODs usually protect catalases and peroxidases against superoxide radicals while being protected by them against H_2O_2 (38). Therefore, the decrease in SOD activity in the lungs of anilide-treated animals will lead to the accumulation of superoxide radicals and make them more susceptible to oxidative damage. Martin *et al.* (7) have also reported that lung injury in toxic oil syndrome is due to the generation of free radicals caused by anilides.

Anilide ingestion decreased the activity of NADPH-generating enzymes in the lungs (Table 4). This might explain why we observed a decrease in the activity of GSH-Px and a corresponding increase in the susceptibility of lipids to peroxidation in the lungs of anilides-treated animals (Table 5).

It has been reported that TOS patients develop acute pulmonary edema (10), adult respiratory distress syndrome (40), and lung injury (41). We have also observed pancellular toxicity in the lungs of guinea pigs ingesting oleylanilide (42). SOD protects lungs from vascular injury, and the activity of SOD is decreased in the lungs due to anilides ingestion. Hence, it is possible that superoxide ions, and perhaps other oxygen products, directly participate in the production of lung injury. Another possibility is that superoxide radicals may act indirectly, by generating a chemotactic lipid that amplifies the requirement of neutrophils and thereby

intensifies the inflammatory response (43). Furthermore, oxygen-containing iron radicals (ferryl and perferryl ions) or hydroxy radicals may be the key products involved in lung injury.

Thus, anilides ingestion can precipitate symptoms of lung damage due to overproduction of free radicals through the inhibition of the activities of SOD and GSH-Px. However, it must be pointed out that lung is a complex tissue containing many different cell types. Damage caused by the anilides ingestion may well destroy some cell types and encourage others to proliferate. Therefore, the changes in the enzyme activities as assayed in the whole lung homogenatenates may not reflect the changes in individual cell types. We are currently investigating the effects of anilide consumption on the oxygen defense systems of guinea pig alveolar type II cells.

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