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Mechanisms of Arachidonic Acid In Vitro Schistosomicidal Potential

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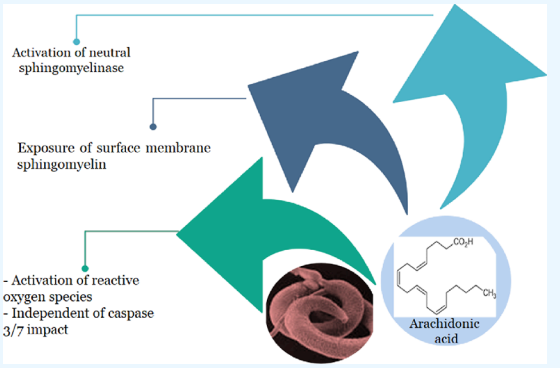
ABSTRACT: Arachidonic acid (ARA) was shown to possess safe and effective schistosomicidal impact on larval and adult *Schistosoma mansoni* and *Schistosoma hematobium* in vitro and in vivo in laboratory rodents and in children residing in low and high endemicity regions. We herein examine mechanisms underlying ARA schistosomicidal potential over two experiments, using in each pool a minimum of 50 adult male, female, or mixed-sex freshly recovered, ex vivo *S. mansoni*. Worms incubated in fetal calf serum-free medium were exposed to 0 or 10 mM ARA for 1 h at 37 °C and immediately processed for preparation of surface membrane and whole worm body homogenate extracts. Mixed-sex worms were additionally used for evaluating the impact of ARA exposure on the visualization of outer membrane cholesterol, sphingomyelin (SM), and ceramide in immunofluorescence assays. Following assessment of protein content, extracts of intact and ARA-treated worms were examined and compared for SM content, neutral sphingomyelinase activity, reactive oxygen species levels, and caspase 3/7 activity. Arachidonic acid principally led to perturbation of the organization, integrity, and SM content of the outer membrane of male and female worms and additionally impacted female parasites via stimulating neutral sphingomyelinase activity and oxidative stress. Arachidonic powerful action on female worms combined with its previously documented ovocidal activities supports its use as safe and effective therapy against schistosomiasis, provided implementation of the sorely needed and long waited-for chemical synthesis.



INTRODUCTION

Schistosomiasis is still prevalent in 78 developing countries in the tropics and subtropics, with the vast majority of infections caused by *Schistosoma mansoni* and *Schistosoma hematobium*. Despite extensive and intensive efforts by the World Health Organization (WHO), an estimated 280,000 deaths are recorded annually, and 800 million people in rural communities, primarily children, are still at risk of the infection.[1,2] Increased access to safe water, improved sanitation, health education, efforts to control the intermediate snail hosts with molluscicides, and widespread, mass preventive chemotherapy programs led in 2020 to only 1/78 and in 2023 to 10/78 countries reporting the absence of human infections.[3] Thus, WHO setting 2030 as the goal for transmission interruption in endemic countries does not seem realistic.[1−3]

It is fortunate that repeat treatments with praziquantel (PZQ), the only drug approved by the WHO for schistosomiasis therapy, may lead to elimination of the infection and eggs are no more detected in stools or urine.[4] However, preventive mass drug administration programs rely on the distribution of a single dose to school children and adults at risk. Indeed, each year, millions of school-aged African children receive PZQ at a standard single dose of 40 mg/kg of body weight.[1−4] On the one hand, total cure of infection is rarely achieved, as "preventive" only implies the commendable goal of reducing morbidity and mortality.[3] On the other hand, infection-free individuals are subjected to treatment,[4] while the term "preventive" in this case is misleading and may promote complacency in hygiene restrictions. Notably, PZQ 40 mg/kg dose does not prevent infection as it is rapidly metabolized in the liver, with a half-life of less than 10 h,[5−9] and thus, may not be available for killing invading larvae within days or weeks. The term "preventive" chemotherapy[3] for the ongoing programs should be revisited, especially that it is documented that PZQ administration does not prevent reinfection even after complete cure.[3,10,11] Additionally, total cure is often not achieved even after multiple treatments,[12,13] which may elicit severe health problems.[4,14,15]

An effective alternative would be to recommend repeat, frequent, if not continuous, intake during the *Schistosoma* transmission season,[16] to prevent development of invading larvae, and single high dose for treatment of established infection, not with a drug but a remedy, even better, a nutrient sorely needed, especially by children of poor rural

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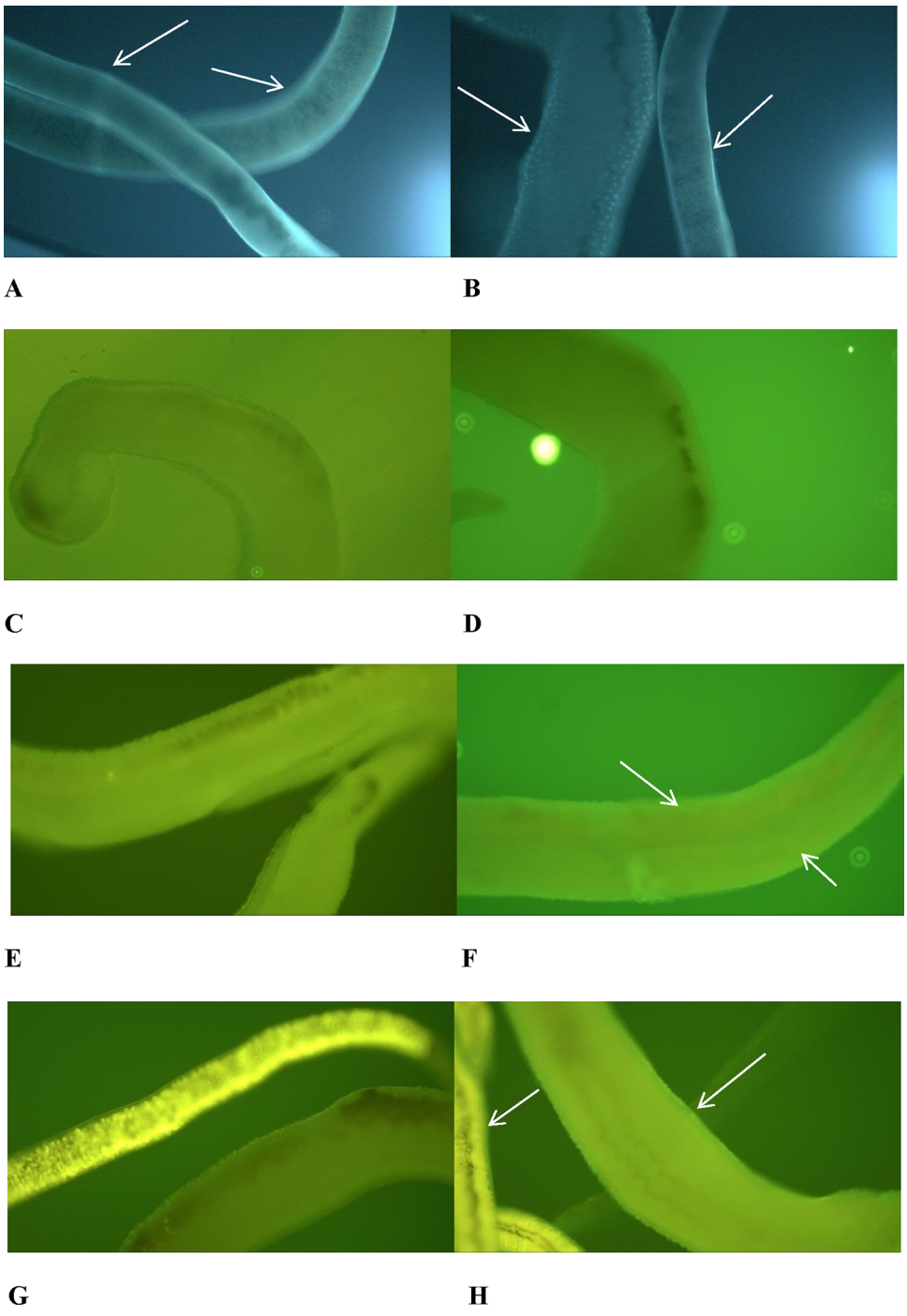

Figure 1. Effect of ARA treatment on adult *S. mansoni* surface membrane exposure of cholesterol, sphingomyelin, and ceramide. Intact (A) and ARA-treated (B) worms show reactivity to filipin in direct membrane immunofluorescence. Intact (C, D, G) and ARA-treated (E, F, H) worms were incubated in the presence of 0 (C, E) or 3 (D, F) μg/mL lysenin or 1:50-diluted mouse monoclonal antibody to ceramide (G, H) in indirect membrane immunofluorescence. (×200). Arrows point to typical worm strong reactivity recorded over two independent experiments.

communities: arachidonic acid (ARA). Arachidonic acid was found to display the strongest impact against larval and adult *S. mansoni* and *S. hematobium* when compared to oleic and linoleic acid in corn and olive oil and >98% pure palmitic, linoleic, and docosahexaenoic acid and phosphatidyl serine.[17−19] Arachidonic acid schistosomicidal potential against lung-, liver-, and adult-stage *S. mansoni* and *S. hematobium* has been documented in vitro and in vivo in rodents.[17,18,20−23] Lung-stage schistosomula and liver and small intestine-derived parasite eggs[22,23] are sensitive to as low as 50 μM ARA = 15 μg/mL, the most physiological and easily attained human serum level.[24,25] For complete cessation of movement and death, adult worms require 30 to 60 min exposure to up to 10 mM ARA,[20] a level difficult to reach even in the portal circulation. However, adult worms may succumb to significantly lower serum ARA levels if the exposure is continuously maintained, for days or weeks. In support, providing school-age children with 10 mg/kg ARA daily for 15 days over 3 weeks (5 days/week) was as effective as PZQ regarding cure rates and egg reductions of light intensity (<100 eggs per gram stool,

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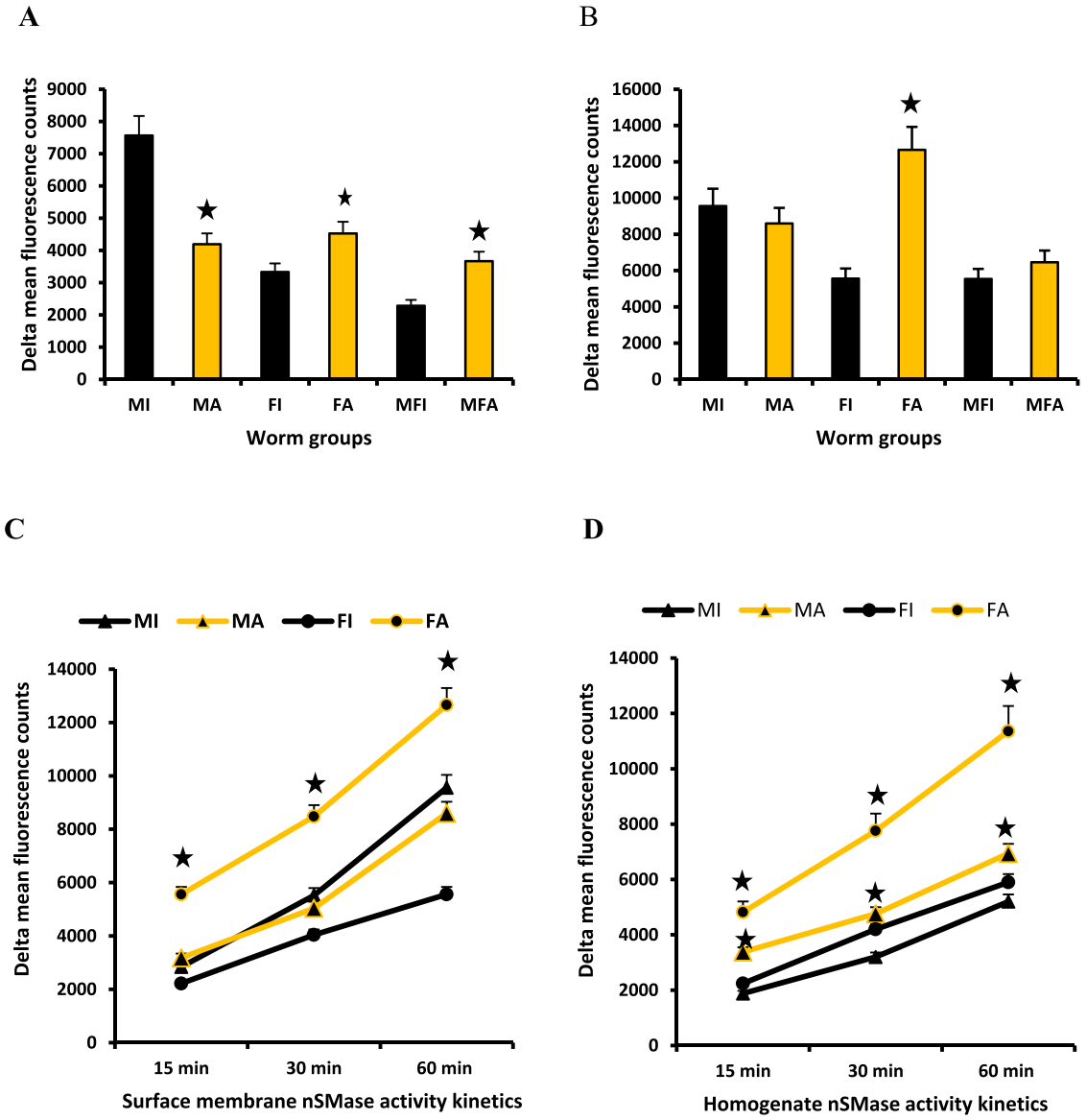

Figure 2. Surface membrane and homogenate extract nSMase activity. Results of two independent experiments, using pools each of 50 ± 5 six week-old *S. mansoni* worms, are depicted (Experiment 1, A; Experiment 2, B–D), showing mean delta (background counts subtracted) fluorescence counts reflecting nSMase activity in 20 μg/well Triton X-100-extracted surface membrane (A, B, C) or homogenate (D) proteins of intact (black columns and lines) male (MI), female (FI), and mixed-sex (MFI), and ARA-treated (yellow-brown columns and lines) male (MA), female (FA), and mixed-sex (MFA) worms. Vertical lines represent the SE around the mean of duplicate wells of two assays. Differences of nSMase activity values between same-sex intact and ARA-treated worms were analyzed by a two-tailed paired *t* test. Significant values ($P < 0.05$) are indicated by asterisks.

epg) infections. Complete cure of moderate (100–400 epg) or heavy (>400 epg) infections was not achieved by PZQ or ARA alone; however, a single PZQ dose and 15 day ARA treatment led to 100% and 78% cure rates of moderate and heavy infections, respectively.[26,27] The safety of ARA administration was remarkable, with not a single adverse reaction noted or recorded. Nevertheless, complete elimination of worms in heavy infection was not achieved upon use of ARA alone or in combination with PZQ because exposure time is a rate-limiting factor for ARA (and PZQ) impact on adult schistosomes. Blood flukes may be attacked uniquely in the circulation, where ARA and PZQ are subject to hepatic first-pass metabolism and clearance from plasma, leading to recommending the highest possible dosages for maximum efficacy.[5−9] This caveat is more serious for ARA due to ready binding to serum albumin.[17] Using high doses is inevitable for expressing ARA schistosomicidal potential and is in accord with the entire safety of dietary or supplementary ARA.[28,29] Therefore, exposure to 10 mM ARA for a maximum of 60 min was used to clarify in vitro the mechanism(s) underlying the ARA schistosomicidal effect on adult male and female *S. mansoni*. Data obtained with mixed-sex worms consistently corroborated and confirmed the robustness of the findings. Arachidonic acid exposure led to disturbing the outer membrane organization and promoted conversion and reduction of the sphingomyelin (SM) content of male and female worms. The further powerful impact of ARA exposure on female worms' enzyme activity and reactive oxygen species production supports advocating it as schistosomicide of the future.

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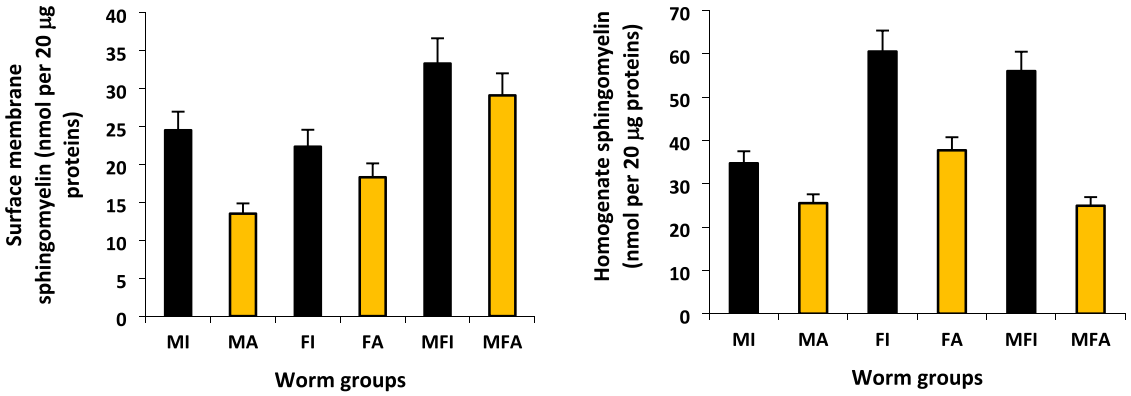

Figure 3. Effect of ARA treatment on the worm sphingomyelin content. Typical of two independent experiments. Each column represents the mean SM content in 20 μg proteins/well, and vertical bars depict the SE around the mean of duplicate wells. All data passed the normality tests. The surface membrane and homogenate SM content in ARA-treated worms was consistently lower than that in intact worms, but the differences were not significant.

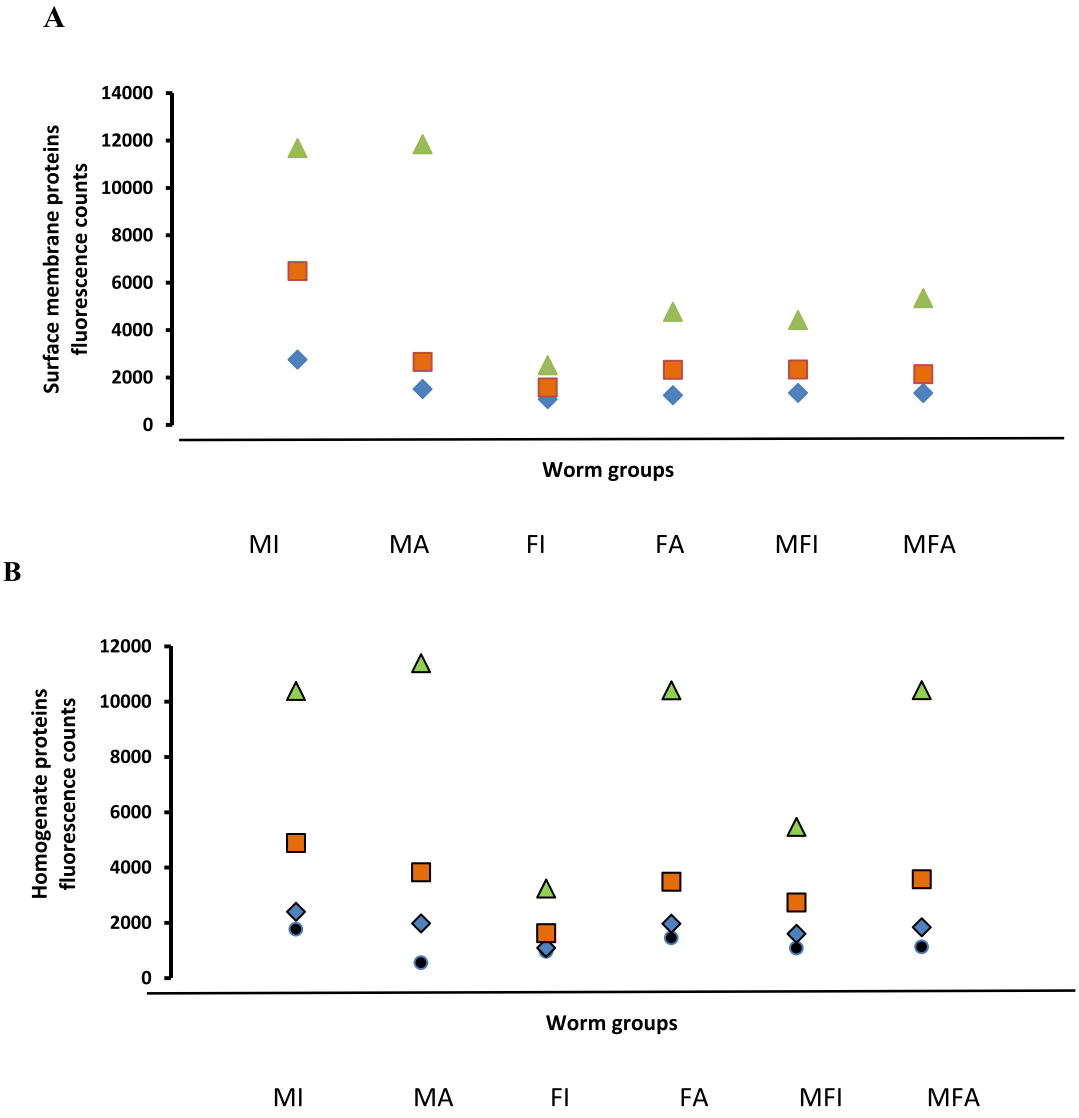

Figure 4. Effect of arachidonic acid treatment on worm reactive oxygen species content. Each point represents mean delta (background fluorescence subtracted) fluorescence counts of surface membrane (A) and homogenate (B) 5 (black circles) and/or 10 (blue diamonds), 20 (brown squares), and 40 (green triangles) μg/well protein of pools each of 50 ± 5 intact male (MI), female (FI) and mixed-sex (MFI) and ARA-exposed male (MA), female (FA) and mixed sex (MFA) six week-old worms, 1 h after incubation with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate. Results are typical of two independent experiments.

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RESULTS

Surface Membrane Cholesterol, Sphingomyelin, and Ceramide Visualization. Cholesterol-filipin fluorescence was evident on the surface of intact and ARA-treated male and female worms (Figure 1A,B), while specific lysenin (Figure 1C−F) and antibody to ceramide (Figure 1G,H) binding was only observed on the surface of ARA-treated worms with all male and female worms in duplicate wells scored as positive.

Effect of ARA Treatment on Neutral Sphingomyelinase Activity. Results of repeat experiments revealed that neutral sphingomyelinase (nSMase) activity in surface membrane extracts of intact male was consistently higher than female worms (Figure 2A−C), while the opposite was recorded for nSMase activity in homogenate extracts (Figure 2D); yet, differences in fluorescence counts were not or barely significant. Arachidonic acid treatment differentially impacted the nSMase activity of male worms, eliciting a remarkable decline and significant ($P < 0.05$) increase in nSMase activity of surface membrane and homogenate extracts, respectively (Figure 2A−D). Contrary to males, female worm nSMase activity in surface membrane and homogenate extracts was readily ($P < 0.05$) activated following 1 h ARA exposure (Figure 2A−D; Table S1).

Sphingomyelin Content. The content of SM in the surface membrane and homogenate extracts of male and female worms did not significantly differ. The sphingomyelin content of intact worms' surface membrane was consistently lower than that of homogenates extracts, but the differences were only barely significant. Arachidonic acid treatment led to a decrease in surface membrane and homogenate SM contents in males and females compared to intact worms, but the differences were not significant (Figure 3).

Reactive Oxygen Species. Repeat experiments revealed significantly higher (P <0.01 to <0.001) ROS content in surface membrane and homogenate extracts of intact male compared to intact female worms, further supported by the intermediate fluorescence counts of mixed-sex worms (Figure 4, Table 1). Arachidonic acid treatment elicited a significant decrease in ROS level in surface membrane ($P < 0.01$) and homogenate ($P < 0.05$) 10 and 20 μg/well proteins of male worms. The inhibition was not evident upon testing 40 μg/well proteins. Conversely, ARA treatment elicited a significant increase in ROS release in surface membrane and homogenate 10, 20, and 40 μg/well (P <0.01 to <0.001) proteins of female worms (Table S1). The ROS fluorescence counts of mixed-sex worms were in accord with the differential ARA impact on ROS activity in male versus female worms (Figure 4, Table 1). Estimation of ROS reactivity in total worm body homogenate (20 μg proteins/well) provided powerful support for the data recorded over two independent experiments (Figure S1).

Hydrogen peroxide released by 20 μg extract proteins (per well) varied between 83 and 88 pmol with SE among assays and wells of <1%. Yet, the data replicated the findings recorded with total ROS regarding differences between intact male and female worm extracts and ARA differential impact on male and female worms (Figure 5; Table S1).

Values of nitric oxide products released by 20 μg extract proteins (per well) replicated the findings recorded with total ROS regarding differences between intact male and female worms' extracts and ARA significant ($P < 0.05$, Figure S2) impact on female worms, except for the lack of a significant decrease in male worm surface membrane and homogenate extracts (Figure 6 and Figure S2; Table S1).

Table 1. Effect of Arachidonic Acid Treatment on Worm Reactive Oxygen Species[a]

	Mean delta fluorescence counts ± SE		
Extract	10 μg/well	20 μg/well	40 μg/well
Worm group			
	Surface Membrane		
MI	2750 ± 68	6490 ± 137	11677 ± 455
MA	1513 ± 73	2655 ± 89	11838 ± 1015
P	0.0064	0.0007	NS
FI	1085 ± 19**	1575 ± 55***	2509 ± 10**
FA	1251 ± 75	2322 ± 12	4770 ± 67
P	NS	0.0056	0.0009
MFI	1346 ± 10	2331 ± 45	4423 ± 85
MFA	1337 ± 58	2139 ± 21	5346 ± 109
P	NS	NS	0.0217
Homogenate			
MI	2402 ± 34	4889 ± 47	10390 ± 105
MA	1979 ± 58	3828 ± 137	11390 ± 266
P	0.0243	0.0181	NS
FI	1095 ± 45***	1626 ± 18***	3240 ± 14***
FA	1969 ± 55	3487 ± 67	10412 ± 412
P	0.0069	0.0014	0.0033
MFI	1605 ± 25	2737 ± 59	5472 ± 95
MFA	1842 ± 56	3578 ± 50	10421 ± 56
P	0.05	0.0084	0.0005

[a]Surface membrane and homogenate Triton X-100-extracts of pools each of 50 ± 5 intact male (MI), female (FI) and mixed sex (MFI) and ARA-exposed male (MA), female (FA) and mixed sex (MFA) six week-old worms were assayed for release of reactive oxygen species 1 h after incubation with 20 μM 2′,7′-dichlorodihydrofluorescein diacetate. Delta = background fluorescence subtracted. Mean of 2 assay wells/experiment and SE about the mean are shown. Differences of values were assayed using two-tailed *t* test. *P* values show differences between intact and ARA-treated worms. NS = not significant. Asterisks indicate statistical differences between intact male and intact female worms, ** $P < 0.01$; *** $P < 0.001$.

Lipid Peroxidation Product. The lipid peroxidation assay showed that a higher ROS content in intact male worms compared to female worms failed to enhance lipid peroxidation. However, ARA exposure increased the level of the lipid peroxidation biomarker in male and not in female worms (Figure 7, Table S1).

Caspase 3/7 Activity. Caspase 3/7 activity differed between intact worm surface membrane and homogenate extracts and between male compared to female worms. Caspase activity was significantly lower in 10 ($P < 0.01$), 20 ($P < 0.001$) and 40 ($P < 0.0001$) μg/well surface membrane proteins of male compared to female worms. Conversely, caspase activity was significantly higher in 10, 20, and 40 ($P <$ 0.01) μg/well homogenate proteins of male compared to female worms (Table S1). Mixed-sex worms consistently showed intermediate luminescence counts (Figure 8; Table 2). Arachidonic acid treatment led to a significant (P <0.001 to <0.0001) decrease in caspase 3/7 activity in surface membrane and homogenate extracts of male, female, and mixed-sex worms (Figure 8; Table 2; Table S1).

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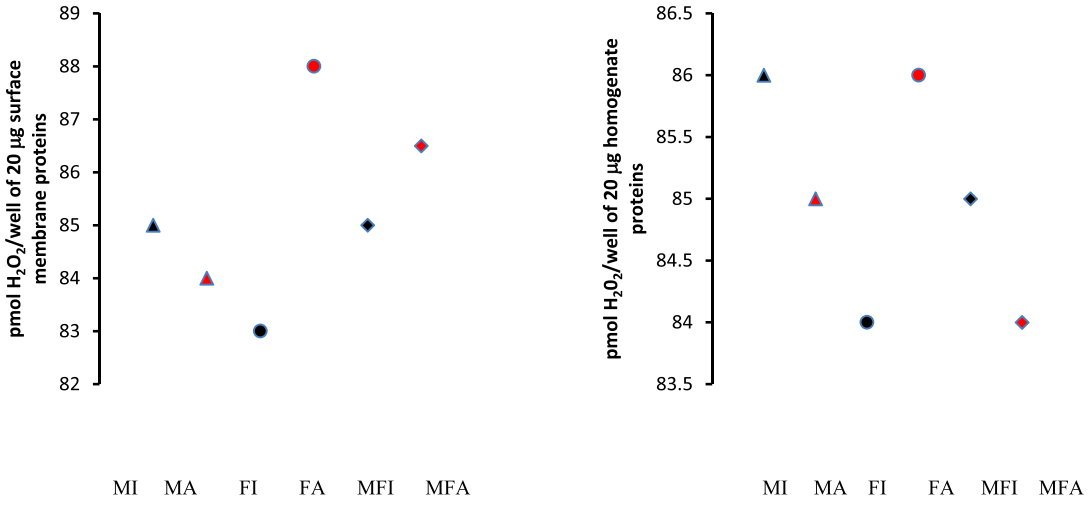

Figure 5. Effect of arachidonic acid treatment on the worm extract hydrogen peroxide content. Each point represents mean released pmol H_2O_2 per well of 20 μg surface membrane (left panel) and homogenate (right panel) proteins of pools each of 50 ± 5 intact male (MI), female (FI) and mixed-sex (MFI) and ARA-exposed male (MA), female (FA) and mixed-sex (MFA) 6 week-old worms. Results are the mean of two independent experiments, with SE less than 1%.

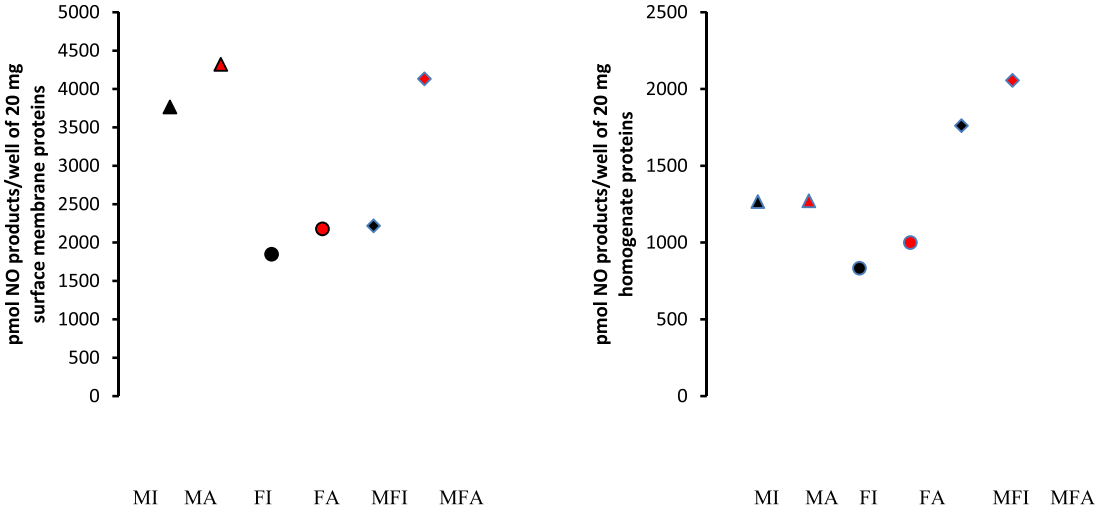

Figure 6. Effect of arachidonic acid treatment on the worm nitric oxide product content. Each point represents mean released pmol of NO products per well of 20 μg of surface membrane (left panel) and homogenate (right panel) proteins of pools each of 50 ± 5 intact male (MI), female (FI) and mixed-sex (MFI) and ARA-exposed male (MA), female (FA) and mixed-sex (MFA) 6 week-old worms. Values are mean of two independent experiments with SE < 5%.

DISCUSSION

In vitro treatment of lung-stage *S. mansoni* and *S. hematobium* schistosomula with 50 μM ARA for 1 h elicited exposure of their otherwise concealed surface membrane molecules to antibody binding and eventual death.[17,18] Developing and adult *S. mansoni* worms are irreversibly dead after 3 to 5 h in vitro exposure to 2.5 and 5.0 mM but are not sensitive to the physiological 1 mM ARA (0.3 mg/mL) concentration.[20,30] Supplementary free ARA half-life in serum is limited due to binding to albumin and retention in the liver.[30] Therefore, one or two high, but safe,[31,32] ARA dose(s) of 3600 mg should be recommended for attaining the efficacious 5.0 to 10 mM concentration for at least 1 h, in the portal and mesenteric vessels. In an aim to examine putative in vivo ARA schistosomicidal mechanisms, 6 week-old *S. mansoni* freshly recovered from infected hosts were exposed to 0 or to 10 mM ARA at 37 °C for exactly 1 h. Surface membrane filipin-cholesterol fluorescence was not different among intact and ARA-treated adult worms, corroborating the findings obtained with *S. mansoni* and *S. hematobium* lung-stage schistosomula that reveal limited ARA impact on cholesterol visualization in the outer lipid bilayer.[17] Conversely, ARA treatment elicited exposure of otherwise concealed SM clusters and ceramide, likely via disruption of the hydrogen bonds barrier, which surrounds the worm and uniquely allows small molecules like ARA (304.47 g/mol = 304.47 Da) and filipin III (654.8 Da), but not the 33 kDa lysenin or antibody to ceramide, to access the apical membrane molecules.[18,33−35]

The nSMase specific activity in surface membrane extracts was remarkably higher in untreated intact male compared to female worms, while nSMase activity in homogenates was higher in females compared to males, point by point corroborating data previously recorded.[19] Notably, cathepsin B and L specific activities in total body extracts of female *S. mansoni* were remarkably higher than those in extracts of male worms.[36] Transcript levels of the gene encoding tegument

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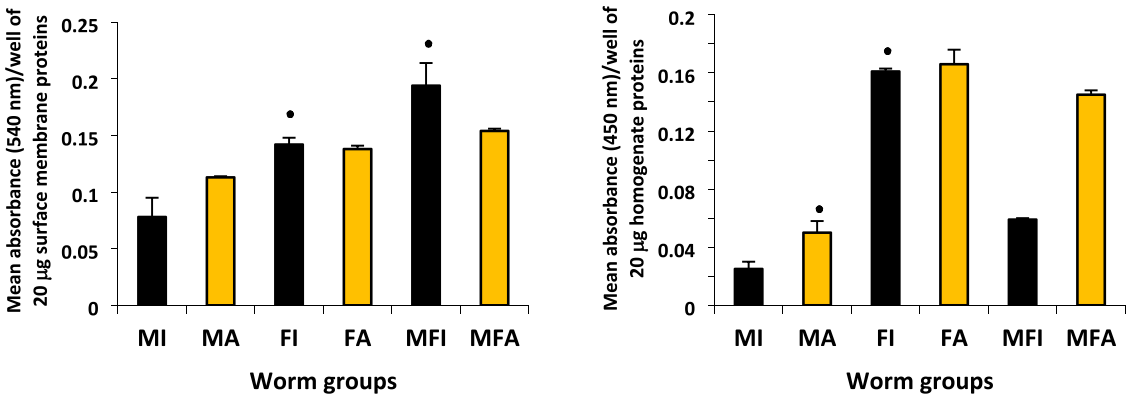

Figure 7. Effect of arachidonic acid treatment on the worm lipid peroxidation status. Each point represents mean absorbance per well of 20 μg surface membrane (left panel) and homogenate (right panel) proteins of pools each of 50 ± 5 intact male (MI), female (FI) and mixed-sex (MFI) and ARA-exposed male (MA), female (FA) and mixed-sex (MFA) 6 week-old worms. Column values are the mean of replicate wells of two independent experiments, and vertical bars denote SE around the mean. Asterisks indicate significant differences ($P < 0.05$) compared to intact male worms.

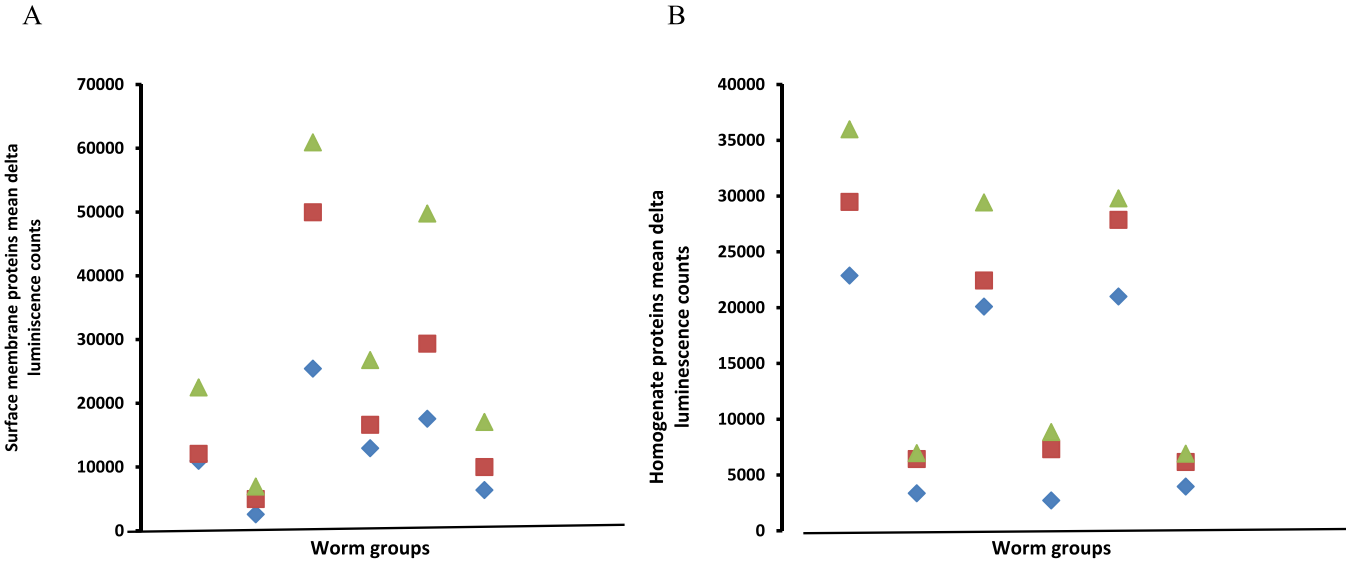

Figure 8. Effect of arachidonic acid treatment on worm caspase 3/7 activity. Each point represents mean delta (background fluorescence subtracted) luminescence counts of surface membrane (A) and homogenate (B) 10 (blue diamonds), 20 (brown squares), and 40 (green triangles) μg/well proteins of pools each of 50 ± 5 intact male (MI), female (FI) and mixed sex (MFI) and ARA-exposed male (MA), female (FA) and mixed-sex (MFA) 6 week-old worms, 1 h after incubation with Caspase-Glo 3/7 reagent. Results are typical of two independent experiments.

aldehyde dehydrogenase, SmALDH_312, differed between male and females Liberian strain *S. mansoni* worms.[37] Our findings are thus in accord with the considerable differences in protein content and activity between male and females recorded in *S. japonicum*, *S. mekongi*, and *S. mansoni*[36−42] and additionally reveal the distinct properties of the worm surface tegument and body. High surface membrane nSMase activity could be deleterious, as it is readily increased after exposure to the high levels of unsaturated fatty acids in the portal and mesenteric circulation.[24,25,30,43−45] Excessive nSMase activation will lead to SM hydrolysis, surface membrane disintegration, and worm death. Strangely, nSMase activity in surface membrane extracts of male worms significantly declined following exposure to ARA, which considerably increased the nSMase activity in the surface membrane and homogenate extracts of female worms. The findings together reveal the vulnerability of the female worms compared to the male worms and indicate that nSMase activation may provide a basis for ARA schistosomicidal impact on female worms.

The sphingomyelin content (nmol/20 μg protein) was rather similar in surface membrane extracts of intact male and female worms, but was lower than in homogenate extracts in accord with the documented differences in lipidome between tegument and whole worm body.[46] The higher SM content in female and mixed-sex worm's homogenates may be attributed to the additional SM content of eggs in the uterus.[47] The sphingomyelin content in surface membrane and homogenate extracts of male, female, and mixed-sex worms was consistently decreased following exposure to ARA. Redman et al.[48] have shown that *S. mansoni* adult worms are able to breakdown SM to ceramide, an activity stimulated by ARA and magnesium chloride. A decrease in SM content will lead to perturbation of the outer lipid organization and integrity, while accumulation of the pro-apoptotic ceramide may be lethal to the worms, mechanisms likely underlying the ARA schistosomicidal potential.

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Table 2. Effect of Arachidonic Acid Treatment on Worm Caspase 3/7 Activity[a]

	mean delta luminescence counts ± SE		
extract	10 μg/well	20 μg/well	40 μg/well
Worm group			
Surface Membrane			
MI	10974 ± 965	12063 ± 748	22477 ± 199
MA	2582 ± 209	4968 ± 283	6936 ± 129
P	0.0011	0.0013	<0.0001
Percent decrease	76	58	69
FI	25420 ± 2762*	49956 ± 2915**	60923 ± 710***
FA	12947 ± 2201	16630 ± 3056	
P	0.0242	0.0014	
Percent decrease	49	66	56
MFI	17571 ± 1266	29352 ± 1919	49768 ± 367
MFA	6388 ± 762	9986 ± 634	17058 ± 238
P	0.0016		0.0030
Percent decrease	63	66	65
MI	10974 ± 965	12063 ± 748	22477 ± 199
MA	2582 ± 209	4968 ± 283	6936 ± 129
P	0.0011	0.0013	<0.0001
Percent decrease	76	58	69
Homogenate			
MI	22873 ± 3102	29482 ± 1153	35984 ± 754
MA	3361 ± 365	6434 ± 993	6958 ± 345
P	0.0247	0.0006	0.0007
Percent decrease	85	78	80
FI	20089 ± 2540	22436 ± 1263*	29425 ± 345*
FA	2712 ± 218	7312 ± 896	8842 ± 140
P	0.0209	0.0006	0.0003
Percent decrease	86	67	70
MFI	21100 ± 2189	27862 ± 2189	29793 ± 403
MFA	3957 ± 336	6166 ± 714	6901 ± 951
P	0.0042	0.0114	0.0020
Percent decrease	81	77	76

[a]Surface membrane and homogenate extracts of a pool each of 50 ± intact male (MI), female (FI) and mixed-sex (MFI) and ARA-exposed male (MA), female (FA) and mixed-sex (MFA) six week-old worms were assayed for caspase 3/7 activity, 1 h after incubation with reagent. Delta = background luminescence subtracted. Mean of duplicate wells of 2 assays and SE about the mean are depicted. Differences of values were analyzed using two-tailed *t* test. *P* values show differences between intact and ARA-treated worms. NS = not significant. Asterisks indicate statistical differences between intact male and intact female worms, * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. Percent decrease = mean intact worms counts−mean ARA-treated worms counts/mean intact worms counts. Results are typical of two independent experiments.

The reactive oxygen species content was evaluated in worm extracts as early as possible after recovery from hamsters or ARA treatment as recommended, while efforts were made to decipher whether the measured ROS activity involves hydrogen peroxide and nitric oxide radicals.[49,50] Untreated intact males strikingly differed from females in harboring significantly (*P* <0.001 to <0.0001) higher ROS, including H_2O_2 and NO, activity, likely because male *S. mansoni* are far more exposed to extrinsic factors than the females lodged in the gynecophoric groove. Yet, male worms appear to have developed effective arms to counteract ROS stimulants as ROS activity in 5, 10, and 20 μg proteins significantly (*P* <0.02 to <0.001) declined after ARA treatment, which conversely led to significant ($P < 0.01$) stimulation of ROS activity in surface membrane and homogenate extracts of female worms. Additionally, a high ROS content in male versus female worms failed to translate into an increase in lipid peroxidation, which appeared to plague female worms (Figure 7). The findings together indicate that male worms are resistant to oxidative stress, which results from imbalance between ROS accumulation and elimination.[51,52] Antioxidant enzymes play a major role in antioxidant defense. Resistance to ARA-mediated oxidative impact in males may be attributed to the higher expression of antioxidant enzymes and defenses compared to female worms. In support, several antioxidant enzymes, superoxide dismutase, and members of the glutathione and thioredoxin systems, counteracting host ROS have been identified in schistosomes, with adult worms showing higher enzyme protein expression compared to larval stages and gender differences were reported.[51−59] Accordingly, an increase in ROS production may be considered a potent mechanism for ARA schistosomicidal impact on female, but not male, *S. mansoni*.

Caspase 3 and 7 are structurally similar and play a critical role in mitochondria outer membrane permeabilization and loss of membrane potential-related apoptosis events and have been termed apoptotic executors.[60−62] High caspase 3 activity in intact females may give an explanation for the low ROS levels as it is documented that caspase 3 inhibits ROS production.[63] Caspase 3 inhibition by ARA was associated with an increase in ROS levels in the surface membrane and homogenate extracts of female worms. Low caspase 3 activity in intact males might be attributed to the inhibitory impact of high ROS levels.[64] Caspase 3 and 7 were active in each developmental stage of *Schistosoma japonicum* but were highest in the 14 days post infection schistosomula than in adults.[65] For a change, surface membrane and whole body homogenate extracts of males and females uniformly displayed a highly significant reduction in caspase 3/7 activity following exposure to ARA. The data thus indicate that ARA schistosomicidal action is independent of caspase 3 and caspase 7-related apoptotic events.

CONCLUSIONS

Taken together, the findings revealed significant differences between males and females in *S. mansoni* and between the surface membrane and whole-body worm extracts in the enzyme, notably nSMase and caspase 3/7, activity, and SM and ROS content. Arachidonic acid affected male and female viability via exposure of otherwise concealed SM and ceramide and additionally impacted female worms via nSMase activation and an increase in ROS levels. The study provides support for advocating ARA as the schistosomicide of the future provided promoting user-friendly protocols for ARA production from fungi and algae[66] and chemical synthesis.[67]

MATERIALS AND METHODS

Adult Worms. Over two independent experiments, 6 week-old worms were obtained from male hamsters infected with Egyptian strain *Schistosoma mansoni* cercariae at the Schistosome Biological Supply Program-Theodore Bilharz

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Research Institute. The worms were placed immediately upon perfusion in sterile Roswell-Park Memorial Institute 1640 (RPMI) medium supplemented with 5% fetal calf serum (FCS). Paired male and female worms were separated using gentle procedures and randomly distributed into pools of 50 ± 5 worms, two for each of male, female, and mixed-sex worms, which were then incubated at 37 °C for 1 h in sterile FCS-free RPMI medium to regenerate from any handling impact.

Arachidonic Acid Treatment. Stock of 500 mg arachidonic acid/mL ethanol (Item No. 90010, purity >98%; Cayman Chemical, Neratovice, Czechia) was divided upon arrival into 50 μL aliquots, which were immediately stored at −20 °C and used only once to avoid oxidation. Groups of 6 week-old 50 ± 5 male (M), female (F), or mixed-sex (M) worms were incubated in 2 mL of FCS-free medium in wells of sterile six well-plates and exposed for 1 h at 37 °C to 0 or 10 mM (3 mg/mL). Untreated worms remained intact, viable, and contractile. All ARA-treated worms largely lost movement and contractility within 30 min and were entirely immobile and irreversibly dead after 1 h but without overt evidence of surface membrane disintegration as examined by ×200 light microscopy. All worms were washed 3× in medium to remove ARA and ethanol traces before processing for the different assays.

Surface Membrane Cholesterol, Sphingomyelin, and Ceramide Visualization. Aliquots of 5 to 8 mixed-sex worms in replicate wells of 48-well plates were used to examine the effect of ARA treatment on worms' surface membrane cholesterol, sphingomyelin (SM), and ceramide localization in immunofluorescence assays (IF).

Cholesterol. Worms were incubated for 10 min at room temperature with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline, pH 7.1 (D-PBS), washed 3× in D-PBS, and exposed for 2 h at room temperature to 50 μM Filipin III (Sigma-Aldrich, St. Louis, MO, USA), a fluorescent polyene macrolide antibiotic, widely used to probe cholesterol location in biological membranes.[68] After washing in D-PBS, filipin-cholesterol fluorescence was visualized by ultraviolet (UV) microscopy using an Olympus inverted microscope (Tokyo, Japan) and UV filter model U-MWU2.[69,70]

Sphingomyelin. Paraformaldehyde-fixed worms were thoroughly washed in FCS-free medium and incubated overnight at room temperature in the presence of 0 or 3 μg/mL lysenin (Sigma). Lysenin, a protein derived from the coelomic fluid of the earthworm *Eisenia fetida*, recognizes the SM heterogeneous organization in bio-membranes via specific binding to clusters of 5−6 SM molecules.[33] After washing, control and lysenin-exposed worms were incubated with 1:100-diluted rabbit antibodies to lysenin (MyBioSource, San Diego, CA, USA), washed 3× and incubated with 1:100-diluted fluorescein isothiocyanate (FITC)-labeled antibodies to rabbit immunoglobulins (Sigma), and inspected by alternate light and UV microscopy.[71]

Ceramide. Paraformaldehyde-fixed worms were incubated overnight at room temperature in the presence of 1:20-diluted mouse monoclonal antibody to ceramide (Sigma- Aldrich-Merck). After washing in medium/1% FCS, all worms were incubated for 1 h at room temperature with 1:50-diluted FITC-labeled antibody to mouse IgG (H+L) (Sigma), examined, and photographed under light and ultraviolet microscopy.[71]

Preparation of Worm Extracts. Groups of 50 ± 5 intact (I) and ARA-treated (A) male, female, and mixed-sex worms were incubated for 30 min on ice with continuous shaking in 0.5 mL of D-PBS/0.1% Triton-X-100 (Promega, Madison, WI, USA) supplemented with protease inhibitors: 2 mM phenyl methyl sulfonyl fluoride and 2 μg/mL leupeptin (Sigma), followed by 10 min at room temperature and 1 min gentle vortexing.

Surface Membrane Extracts. The D-PBS containing Triton-soluble and insoluble surface membrane molecules[33,71,72] was aspirated, assessed for protein content, distributed into aliquots to be maintained at −20 °C, and thawed only once.

Homogenate Extracts. The sedimented worms were suspended in 1.0 mL of D-PBS supplemented with protease inhibitors, thoroughly homogenized on ice for 30 min, and sedimented by centrifugation at 400*g* for 10 min. The supernatant was assessed for protein content, distributed into aliquots to be maintained at −20 °C, and thawed only once.

The sedimented worms' homogenate was also assessed for protein content and used when final confirmation of results was required.

Neutral Sphingomyelinase Activity. Duplicate 20 μg protein aliquots of surface membrane and homogenate extracts of untreated and ARA-exposed worms were evaluated for nSMase activity using a Sphingomyelinase Assay Kit of Abcam, ab287874 (Cambridge Biomedical Campus, Cambridge, United Kingdom), following the manufacturer's instructions.[71] Fluorescence was measured at Ex/Em of 540/590 nm at 15, 30, and 60 min after adding reagents, and counts were shown after subtracting mean background values (Victor X4Multi-Label Plate Reader, PerkinElmer, Waltham, MA, USA).

Sphingomyelin Content. The sphingomyelin content was measured in surface membrane and homogenate extracts of untreated and ARA-treated worms (20 μg protein/well in duplicates) using the Sphingomyelin Assay Kit (Colorimetric), ab287856, (Abcam), following the manufacturer's instructions. Absorption of the standards and test wells was evaluated at 570 nm.

Reactive Oxygen Species. Aliquots of 5, 10, 20, and 40 μg (in 100 μL of D-PBS, pH 7.1) of surface membrane and homogenate extracts proteins of intact and ARA-treated worms were incubated for 1 h at room temperature in the dark with 20 μM 2′,7′-dichlorodihydro-fluorescein diacetate (DCHF-DA), which is oxidized by several ROS to the fluorescent product 2′,7′-dichlorofluorescein.[49,50,73−75] Reactive oxygen species (ROS) release was estimated by fluorescence spectroscopy at Ex/Em 485/535 nm, respectively (Victor X4Multi-Label Plate Reader). Total homogeneous proteins (20 μg/well in duplicates) of intact and ARA-treated worms were also assayed for ROS release.

Reactive oxygen species is a generic term for molecular oxygen and nitrogen-derived oxidants, notably hydrogen peroxide, and nitrogen dioxide radical, which undergoes addition reactions, producing nitrated products.[51,52] Levels of H_2O_2 in Triton X-100 surface membrane and homogenate extract proteins (20 μg/well in duplicates) of intact and ARA-treated worms were evaluated following the instructions of the manufacturer of Hydrogen Peroxide Assay Kit (BioVision, Abcam). In the presence of horseradish peroxidase, the OxiRed probe reacts with H_2O_2 generating a colored product (maximum absorption at OD 570 nm). Then, 0 to 5 nmol/well H_2O_2 was used to plot a standard curve.

The protocol of the Nitric Oxide Fluorometric Assay Kit of BioVision-Abcam was followed to indirectly measure nitric

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oxide release via its composition products, nitrates and nitrites, estimated by fluorescence spectroscopy at Ex/Em 340/450 nm, respectively,[76] in surface membrane and homogenate extracts of intact and ARA-treated worms. Assays for each experiment were performed in duplicate at 20 μg/well in black 96-well microtiter plates (Greiner-Merck). Then, 0 to 1000 pmol/well (in duplicates) nitrate/nitrite standards were used to plot a standard curve.[76]

Lipid Peroxidation Biomarker. Reactive oxygen species lead to oxidative damage of biological macromolecules, notably lipids, resulting in production of hydroperoxides, which fragment to various reactive intermediates, such as malondialdehyde (MDA).[51] The assay of lipid peroxidation is critically important for evaluating the outcome of ROS accumulation.[77] The lipid peroxidation end product, MDA, reacts with thiobarbituric acid (TBA), generating a red adduct, TBA-reactive substances. Therefore, ARA-mediated oxidative impact on worms' fatty acids was estimated via measuring the TBA-reactive substances content in Triton X-100 surface membrane and homogenate extracts (20 μg/well in duplicates) of intact and ARA-treated worms. Briefly, equal volumes of 0.67% thiobarbituric acid (Sigma-Aldrich-Merck) in glacial acetic and 40 μg of worm extract proteins/100 μL of deionized water were incubated for 1 h at 90 °C and distributed after cooling in duplicate wells of microtiter plates (Greiner) for immediate measuring absorbance at OD 540 nm. Similarly treated and measured 1 μM/well 1,1,3,3, tetramethoxypropane (synonym: malonaldehyde bis(dimethyl acetal), Merck) was used as a positive control.[76,78]

Caspase Activity. Caspase 3/7 activity in 10, 20, and 40 μg of surface membrane and homogenate extracts of intact and ARA-treated worms was evaluated using white 96-well plates (Greiner), the Caspase-Glo 3/7 Assay of Promega, and a Victor X4Multi Label Plate Reader. Released luminescence is proportional to the amount of caspase activity present, and luminescent counts are shown after subtracting background values.[60,79,80]

Statistical Analyses. All of the values were tested for normality. Students' *t* test 2-tailed, Mann−Whitney, and one-way ANOVA with post test were used to analyze the statistical significance of differences between selected values, and considered significant at $P < 0.05$ (GraphPad InStat, San Diego, CA, USA).

ETHICS STATEMENT

Experiments involving hamster breeding, infection with cercariae, and worm collection were overseen and approved by the Commission for Evaluation of Animal Use for Research of Theodore Bilharz Research Institute, in strict accordance with the Guide for the Care and Use of Laboratory Animals.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09906.

> Effect of arachidonic acid treatment on worm homogenate reactive oxygen species; effect of arachidonic acid treatment on worm nitric oxide derivatives content; cumulative effects of ARA on nSMase, reactive oxygen species, and caspase 3/7 activity. PDF (PDF)

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H.T.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology; project administration, resources, validation, writing (original draft), writing (review and editing). S.M.: investigation, methodology, writing (review and editing). All authors approved the submission of the revised manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ARA, arachidonic acid; D-PBS, Dulbecco's phosphate-buffered saline, pH 7.1; IF, immunofluorescence; MDA, malondialdehyde; nSMase, neutral sphingomyelinase; PZQ, praziquantel; ROS, reactive oxygen species; SM, sphingomyelin; TBA, thiobarbituric acid; WHO, World Health Organization

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