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ORIGINAL ARTICLE

Aging and Hyperglycemia Intensify Dyslipidemia-Induced Oxidative Stress and Inflammation in Rats: Assessment of Restorative Potentials of ALA and EPA + DHA

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Abstract— Effect of aging and hyperglycemia on oxidative stress (OS) and inflammation in dyslipidemic conditions has not been elucidated. Hence, in this study, we assessed the implications of aging, hyperglycemia, and also the dietary effect of n-3 fatty acids (α-linolenic acid (ALA) and eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)) on OS and inflammation in dyslipidemic rats. Dyslipidemia was induced in young and aged rats by feeding high-fat lard (HFL) diet. Diabetes was induced in young dyslipidemic rats by administering streptozotocin 30 days after the induction of dyslipidemia. Experimental groups received diets containing canola oil (HF + CNO) and fish oil (HF + FO) as a source of ALA and EPA + DHA respectively. After 60 days of feeding rats with their respective diets, OS and inflammatory markers in serum were assessed. Dyslipidemia caused significant ($p < 0.05$) increase in OS (lipid peroxidation, nitric oxide, and protein carbonyl), pro-inflammatory cytokine (CRP, IL-1β, MCP-1, and TNF-α), and eicosanoid (PGE$_2$, LTB$_4$, and LTC$_4$) level in serum of both young and aged rats. Aged dyslipidemic rats presented significantly ($p < 0.05$) higher level of these markers compared to young dyslipidemic rats. Hyperglycemia onset further augmented OS and inflammatory markers in young dyslipidemic rats significantly ($p < 0.05$). Administration of n-3 fatty acids downregulated the serum markers of OS and inflammation in all the three experimental models. Thus, aging and hyperglycemia onset intensified dyslipidemia-induced OS and inflammation. Dietary preformed EPA + DHA presented larger restorative potentials than precursor ALA in countering OS and inflammation in all the three experimental models.

KEY WORDS: n-3 fatty acids; dyslipidemia; hyperglycemia; inflammation; oxidative stress.

INTRODUCTION

Dyslipidemia (hyperlipidemia) and diabetes (hyperglycemia) are the two major risk factors for developing micro- and macro-vascular complications. Studies have shown that their effective management through a healthy diet and physical activity combined with therapeutic intervention may translate into clinical benefits [1]. Metabolism of glucose and lipids are well connected as well as balanced, and any dysregulation in it may result in the clinical manifestation of diabetes and dyslipidemia [2, 3]. Both young and aged populations across the world are susceptible to developing dyslipidemia as well as diabetes. Consumption of excess fat (mainly saturated fat), calories, and sedentary lifestyle along with low intake of bioactive

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lipids could be attributed to the higher incidence of metabolic abnormalities. The occurrence of dyslipidemia individually or in combination with diabetes may elicit a varying degree of oxidative and inflammatory responses. Both OS and chronic inflammation can cause irreversible damage to tissues. The vicious cycle comprising chronic inflammation, OS, and insulin resistance contributes to diabetes-associated complications, including cardiovascular diseases, nephropathy, neuropathy, retinopathy, and urological diseases [4–6]. The extent of disruption in the balance between pro- and anti-oxidants and pro- and anti-inflammatory mediators may determine the severity of the complications [7].

The aging process is characterized by increased OS, heightened inflammatory response, accelerated cellular senescence, and progressive organ dysfunction [8]. The elderly group may show varied responses to dietary factors than younger populations. Comparative evaluation in young and aged models of dyslipidemia and implication of hyperglycemia onset in preexisting dyslipidemic models on OS and inflammatory markers have never been studied. Also, the impact of dietary n-3 fatty acids in modulating oxidative and inflammatory markers in these models needs to be understood for effective application of polyunsaturated fatty acid therapy under metabolically challenged conditions in different age groups. The present study compares oxidative and inflammatory responses in three models, namely, young and aged dyslipidemic rats and young dyslipidemic diabetic rats. The evidence for the restorative potentials of precursor ALA and preformed EPA + DHA against dyslipidemia- and hyperglycemia-induced OS and inflammation are also presented.

MATERIALS AND METHODS

Materials

Ascorbic acid, adenosine diphosphate (1-chloro-2,4, dinitrobenzene), cytochrome C, thiobarbituric acid, xanthine, and xanthine oxidase were obtained from Sigma Chemicals, St. Louis, MO, USA. Malondialdehyde, NADPH, phosphoric acid, sulfanilamide, sulfosalicylic acid, sodium nitrate, t-butyl hydroperoxide, and analytical grade solvents were obtained from SRL Chemicals, Mumbai, India. Food grade commercial brand canola oil and lard were purchased locally, and refined fish oil was procured from Janatha fishmeal products, Udupi, Karnataka, India.

Diet and Animal Feeding

The usage of animals in the experimental protocols was approved by the Institutional Animals Care and Use Committee (IAEC No.311/14). Experiments were carried out in young (22 days/50 ± 5 g) and aged (10 months/350 ± 5 g) male Wistar rats (OUTB-Wistar, IND-cft (2C)) individually housed with 12-h light and dark cycle. Control rats received 7% fat (7 g lard/100 g diet), and high-fat lard (HFL) group received 35% fat (35 g lard/100 g diet). Among the experimental groups, high-fat canola ((HF + CNO), source of ALA, 18:3 n-3) group received 17.5 g lard + 17.5 g canola oil/100 g diet and high-fat fish oil ((HF + FO), source of EPA, 20:5 n-3 + DHA, 22:6 n-3) group received 17.5 g lard + 17.5 g fish oil/100 g diet. To create young dyslipidemic diabetic rat model, after 30 days of feeding HF, HF + CNO, and HF + FO diets, young dyslipidemic rats were made diabetic by injecting streptozotocin (35 mg/kg body weight) and continued with their diet for 30 more days. Upon completion of 60 days of feeding, rats in all the three models were fasted overnight and sacrificed under isoflurane anesthesia. Blood was collected, and serum was isolated by centrifuging the coagulated blood at 10,000 rpm. The collected serum samples were processed as per the analytical requirement and stored at $-80°$ C for further analysis. The dietary fatty acid compositions were measured by gas chromatography as described earlier [9], and the fatty acid profile of diet is given in Table 1. The total n-3 fatty acid content in HF + CNO and HF + FO diets were found to be 33.9 and 22.9% respectively.

Measurement of Oxidative Stress Markers

Serum lipid peroxide level was determined by measuring thiobarbituric acid (TBA) reactivity [10]. Nitric oxide level was measured using the Griess reagent [11]. Protein carbonyls were measured by the method as described by Mesquita *et al.* [12].

Measurement of Inflammatory Markers

Serum IL-1β, MCP-1, and TNF-α were measured by Elisa as per the kit instructions (PeproTech, Rehovot, Israel), and CRP level was measured using the kit purchased from Agappe Diagnostics Ltd., Kerala, India. Serum PGE$_2$, LTB$_4$, and LTC$_4$ were measured by Elisa as per the kit instructions (Cayman Chemicals, USA).

Table 1. Fatty Acid Composition of Diet

Fatty acids (%)	Control	HFL	HF + CNO	HF + FO
14:0	3.8	3.9	1.9	2.1
16:0	25.3	24.8	8.9	4.3
16:1 (n-7)	ND	ND	ND	3.9
18:0	8.8	9.2	3.8	3.1
18:1 (n-9)	44.0	44.3	38.8	36.4
18:2 (n-6)	18.1	17.8	12.7	11.1
18:3 (n-3)	ND	ND	33.9	2.0
20:4 (n-6)	ND	ND	ND	16.2
20:5 (n-3)	ND	ND	ND	11.5
22:6 (n-3)	ND	ND	ND	9.4
$\sum$ n-6	18.1	17.8	12.7	27.3
$\sum$ n-3	NA	NA	33.9	22.9
n-6: n-3	NA	NA	1:2.6	1:0.8

Values are mean of triplicate samples

ND not detected, *NA* not applicable, *HFL* high-fat lard, *HF + CNO* high-fat canola oil, *HF + FO* high-fat fish oil

Statistical Analysis

Results are expressed as mean ± SD. The data were analyzed by one-way ANOVA (non-parametric) followed by Tukey's test. Values in each dietary group between the models [young dyslipidemic (YD) *vs.* aged dyslipidemic (AD) and YD *vs.* young dyslipidemic + diabetic (YDD)] were compared, and a *p* value of < 0.05 was considered statistically significant.

RESULTS

Serum Oxidative Stress Markers

Serum levels of lipid peroxides, nitric oxides, and protein carbonyls indicate the efficiency of systemic antioxidant defense mechanisms. Results from this study indicated significant differences ($p < 0.05$) in the serum level of these markers in YD rats when compared to AD and YDD rats (Table 2). Lipid peroxide level in aged control rats was 72% higher compared to that in young control rats. Dyslipidemia elevated lipid peroxide level in all the three models compared to their respective control group. However, AD and YDD rats showed 25 and 84% higher level of lipid peroxides compared to YD rats. AD and YDD rats fed HF + CNO diet had 37 and 81% higher level of lipid peroxides when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 39 and 72% higher level of lipid peroxides when compared to YD rats fed HF + FO diet. Nitric oxide level in aged control rats was 70% higher compared to that in young control rats. Dyslipidemia elevated nitric oxide level in all the three

models compared to their respective control group. However, AD and YDD rats showed 43 and 79% higher level of nitric oxides compared to YD rats. AD and YDD rats fed HF + CNO diet had 14 and 30% higher level of nitric oxides when compared to YD rats fed HF + CNO diet. Similarly, AD and YDD rats fed HF + FO diet had 35 and 61% higher level of nitric oxides when compared to YD rats fed HF + FO diet. Protein carbonyl level in aged control rats was 42% higher compared to that in young control rats. Dyslipidemia increased protein carbonyl level in all the three models compared to their respective control group. However, AD and YDD rats showed 12 and 45% higher level of protein carbonyl compared to YD rats. AD and YDD rats fed HF + CNO diet had 14 and 47% higher level of protein carbonyl when compared to YD rats fed HF + CNO diet. Similarly, AD and YDD rats fed HF + FO diet had 12 and 46% higher level of protein carbonyl when compared to YD rats fed HF + FO diet.

Serum Cytokine Level

Serum levels of CRP, IL-1β, MCP-1, and TNF-α represent the status of the systemic inflammatory response. Results from this study indicated significant differences ($p < 0.05$) in the serum level of these markers in YD rats when compared to AD and YDD rats (Table 3). Dyslipidemia elevated CRP levels in all the three models compared to their respective control group. However, AD and YDD rats showed 68 and 139% higher level of CRP compared to YD rats. AD and YDD rats fed HF + CNO diet had 23 and 60% higher level of CRP when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had

Table 2. Serum Oxidative Stress Markers

Parameters	Lipid peroxidation (nmol/mg protein)			Nitric oxide (nmol/mg protein)			Protein carbonyl (nmol/mg protein)		
	Model			Model			Model		
Dietary group	YD	AD	YDD	YD	AD	YDD	YD	AD	YDD
Control	6.0 ± 0.9^a	10.3 ± 0.8^b	6.3 ± 1.1^a	4.4 ± 0.4^a	7.5 ± 0.5^b	5.6 ± 0.8^a	5.9 ± 0.6^a	8.4 ± 0.6^b	5.5 ± 0.5^a
HFL	19.5 ± 1.0^a	24.4 ± 0.3^b	35.9 ± 2.1^c	17.7 ± 1.3^a	25.3 ± 0.8^b	31.8 ± 2.0^c	28.7 ± 1.4^a	32.2 ± 1.0^b	41.7 ± 1.6^c
HF + CNO	20.3 ± 0.8^a	27.8 ± 2.2^b	36.9 ± 0.3^c	24.7 ± 1.6^a	28.2 ± 0.8^b	32.3 ± 1.0^c	29.0 ± 2.2^a	33.2 ± 1.7^b	42.8 ± 1.2^c
HF + FO	22.3 ± 0.7^a	31.1 ± 1.1^b	38.4 ± 0.7^c	23.2 ± 0.3^b	31.4 ± 2.1^b	37.4 ± 1.1^c	31.7 ± 2.3^a	35.7 ± 0.8^b	46.5 ± 2.1^c

Values are mean ± SD of 3 rats expressed in nanomoles per milligram protein. Values not sharing a common superscript within the row under each parameter are statistically significant at $p < 0.05$

HFL high-fat lard, *HF + CNO* high-fat canola oil, *HF + FO* high-fat fish oil, *YD* young dyslipidemic, *AD* aged dyslipidemic, *YDD* young dyslipidemic + diabetic

30 and 75% higher level of CRP when compared to YD rats fed HF + FO diet. Dyslipidemia elevated IL-1β levels in all the three models compared to their respective control group. However, AD and YDD rats showed 21 and 44% higher level of IL-1β compared to YD rats. AD and YDD rats fed HF + CNO diet had 13 and 45% higher level of IL-1β when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 12 and 34% higher level of IL-1β when compared to YD rats fed HF + FO diet. Dyslipidemia elevated MCP-1 levels in all the three models compared to their respective control group. However, AD and YDD rats showed 51 and 117% higher level of MCP-1 compared to HFL-fed YD rats. AD and YDD rats fed HF + CNO diet had 30 and 125% higher level of MCP-1 when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 21 and 140% higher level of MCP-1 when compared to YD rats fed HF + FO diet.

Dyslipidemia elevated TNF-α levels in all the three models compared to their respective control group. However, AD and YDD rats showed 24 and 105% higher level of MCP-1 compared to YD rats. AD and YDD rats fed HF + CNO diet had 26 and 138% higher level of TNF-α when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 53 and 142% higher level of TNF-α when compared to YD rats fed HF + FO diet.

Serum Eicosanoid Level

Serum levels of two-series prostaglandin and three-series leukotrienes synthesized from 20 carbon polyunsaturated fatty acids also play a significant role in the sustenance of inflammation. Results from this study indicated significant differences ($p < 0.05$) in the serum level of these lipid inflammatory markers in YD rats when compared to

Table 3. Serum Cytokine Level

Parameters	CRP (mg/dL)			IL-1β (pg/mL)			MCP-1 (pg/mL)			TNF-α (pg/mL)		
	Model			Model			Model			Model		
Dietary group	YD	AD	YDD	YD	AD	YDD	YD	AD	YDD	YD	AD	YDD
Control	1.6 ± 0.2^a	1.6 ± 0.5^a	1.5 ± 0.2^a	5.3 ± 0.7^a	6.0 ± 0.4^a	5.7 ± 0.1^a	3.0 ± 0.1^a	3.2 ± 0.1^a	3.0 ± 0.1^a	1.3 ± 0.2^a	1.6 ± 0.18^a	1.2 ± 0.4^a
HFL	5.0 ± 0.1^a	8.4 ± 0.7^b	11.9 ± 0.5^c	22.1 ± 1.9^a	26.8 ± 1.6^b	31.9 ± 2.8^c	10.1 ± 0.6^a	15.4 ± 1.3^b	22.0 ± 0.4^c	8.1 ± 0.3^a	10.1 ± 0.13^b	16.6 ± 1.0^c
HF + CNO	2.7 ± 0.6^a	3.3 ± 0.6^b	4.3 ± 0.2^c	12.1 ± 0.5^a	13.7 ± 0.2^b	17.6 ± 0.4^c	6.1 ± 0.3^a	8.1 ± 1.2^b	13.9 ± 2.5^c	4.0 ± 0.3^a	5.1 ± 0.2^b	9.7 ± 0.5^c
HF + FO	2.1 ± 0.5^a	2.7 ± 0.7^{ab}	3.6 ± 0.2^b	9.4 ± 0.6^a	10.5 ± 0.4^a	12.5 ± 0.6^b	4.1 ± 0.3^a	5.1 ± 0.2^b	10.0 ± 0.3^c	2.8 ± 0.7^a	4.3 ± 0.7^b	6.8 ± 0.3^c

Values are mean ± SD of 3 rats. CRP is expressed in milligrams per deciliter serum, whereas IL-1β, MCP-1, and TNF-α are expressed in picograms per milliliter serum. Values not sharing a common superscript within the row under each parameter are statistically significant at $p < 0.05$

HFL high-fat lard, *HF + CNO* high-fat canola oil, *HF + FO* high-fat fish oil, *YD* young dyslipidemic, *AD* aged dyslipidemic, *YDD* young dyslipidemic + diabetic

AD and YDD rats (Table 4). Dyslipidemia elevated PGE_2 levels in all the three models compared to their respective control group. However, AD and YDD rats showed 25 and 63% higher level of PGE_2 compared to YD rats. AD and YDD rats fed HF + CNO diet had 25 and 46% higher level of PGE_2 when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 22 and 60% higher level of PGE_2 when compared to YD rats fed HF + FO diet. Dyslipidemia elevated LTB_4 levels in all the three models compared to their respective control group. However, AD and YDD rats showed 29 and 116% higher level of LTB_4 compared to YD rats. AD and YDD rats fed HF + CNO diet had 120 and 210% higher level of LTB_4 when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 53 and 75% higher level of LTB_4 when compared to YD rats fed HF + FO diet. Dyslipidemia elevated LTC_4 levels in all the three models compared to their respective control group. However, AD and YDD rats showed 47 and 153% higher level of LTC_4 compared to YD rats. AD and YDD rats fed HF + CNO diet had 73 and 154% higher level of LTC_4 when compared to YD rats fed HF + CNO. Similarly, AD and YDD rats fed HF + FO diet had 55 and 111% higher level of LTC_4 when compared to YD rats fed HF + FO diet.

DISCUSSION

Though dyslipidemia is well addressed by various researchers, its effect on OS and inflammatory markers in young and aged models has never been comparitively assessed. This is particularly important as metabolic abnormalities that were common to elderly populations are also being diagnosed in younger individuals [13, 14]. Further,

evaluation of the hyperglycemic effect on OS and inflammatory markers under dyslipidemic background may indicate the severity of disease progression among those dyslipidemic individuals who are also prone to hyperglycemia. Considering the differences in the physiological responses for dietary factors among young and elderly populations, we also assessed the implications of dietary n-3 fatty acids in modulating OS and inflammatory markers in all the three models. Dietary n-3 fatty acids are known to possess health beneficial effects [15], and comparing their modulatory effects in these experimental models will potentially exploit its utilization as an adjunct therapy under these metabolically challenged conditions. The oxidative defense mechanism and inflammatory responses are tightly controlled processes, and its dysfunction causes irreversible damages to tissues. One of them manifests before or after the other, but when one manifests, the others most likely appear. The severity of the tissue damage may depend on the cumulative effect of both the processes and also the priming effect of the one that appears first. Hence, the inception of hyperglycemia under preexisting dyslipidemia may pose a greater consequence on the OS and inflammatory markers. Understanding the implications of such co-existing risks on the level of OS and inflammatory markers may aid in designing an effective therapy comprising anti-oxidants and anti-inflammatory molecules. Our earlier studies have demonstrated that dyslipidemia induces dysfunction in the oxidative defense mechanism and regulation of inflammation in young rats [16, 17], while the present study established that aged rats exhibited a higher level of OS and inflammation when compared to younger rats subjected to high-fat induced dyslipidemia for a similar duration.

Further, the onset of hyperglycemia in dyslipidemic rats aggravated the OS and inflammatory markers

Table 4. Serum Eicosanoid Level

Parameters	PGE_2 (ng/mL)			LTB_4 (ng/mL)			LTC_4 (ng/mL)		
	Model			Model			Model		
Dietary group	YD	AD	YDD	YD	AD	YDD	YD	AD	YDD
Control	6.8 ± 0.5^a	8.7 ± 0.5^b	6.4 ± 2.5^a	1.3 ± 0.2^a	1.9 ± 0.03^b	1.3 ± 0.2^a	0.8 ± 0.09^a	1.2 ± 0.1^b	0.8 ± 0.08^a
HFL	18.1 ± 0.7^a	22.6 ± 0.7^b	29.5 ± 0.8^c	3.1 ± 0.10^a	4.0 ± 0.3^b	6.7 ± 0.3^c	1.7 ± 0.2^a	2.5 ± 0.2^b	4.3 ± 0.3^c
HF + CNO	12.9 ± 0.8^a	16.1 ± 0.8^b	18.8 ± 0.7^c	1.0 ± 0.1^a	2.2 ± 0.1^b	3.1 ± 0.2^c	1.1 ± 0.06^a	1.9 ± 0.1^b	2.8 ± 0.2^c
HF + FO	8.5 ± 0.6^a	10.4 ± 0.7^b	13.6 ± 1.1^c	1.2 ± 0.12^a	1.9 ± 0.09^b	2.1 ± 0.2^c	0.9 ± 0.05^a	1.4 ± 0.1^b	1.9 ± 0.2^c

Values are mean ± SD of 3 rats expressed in nanograms per milliliter serum. Values not sharing a common superscript within the row under each parameter are statistically significant at $p < 0.05$

HFL high-fat lard, *HF + CNO* high-fat canola oil, *HF + FO* high-fat fish oil, *YD* young dyslipidemic, *AD* aged dyslipidemic, *YDD* young dyslipidemic + diabetic

compared to the level found in dyslipidemic rats. Metabolic syndrome is characterized by the augmented production of reactive oxygen species and lipid peroxidation resulting in lowered anti-oxidant protection [18]. The induction of hyperglycemia in dyslipidemic rats with the aim to quantify the *per se* effect of hyperglycemia on OS and inflammation revealed that hyperglycemia caused a greater flux in these parameters when compared to only dyslipidemic rats. For instance, the lipid peroxidation of HFL groups in YD and YDD models were higher than that of their corresponding control groups by 225 and 480% respectively, and a similar trend was observed with nitric oxide and protein carbonyl level. Even though the level of lipid peroxides, nitric oxides, and protein carbonyls was found to be higher in the experimental groups fed n-3 fatty acids, the enhanced activity of anti-oxidant enzymes may eventually diminish their deleterious effects [16].

The heightened response by aged rats to diet-induced dyslipidemia may involve immune system remodeling resulting in an increased inflammatory response. The prevalence of chronic low-grade inflammation which is associated with aging is marked by a two- to fourfold increase in serum levels of inflammatory mediators [19]. With increasing age, there is a decrease in the population of dendritic cells and naïve T cells, and to fill the space, as a compensatory mechanism, there will be an expansion of activated T cells and cytotoxic (CD8+) cells [20]. Enhanced macrophage activity can increase the pro-inflammatory cytokine production tilting the balance towards pro-inflammatory milieu [21]. This probably may be the reason for the elevated production of the pro-inflammatory cytokines as well as eicosanoids in the AD rats when compared to the YD rats. Incorporation of n-3 fatty acids (ALA and EPA + DHA) in the diet effectively downregulated the inflammatory eicosanoid and cytokine level in all the three rat models. It was observed that the trend of change in levels of OS markers between YD and AD rat models and also YD and YDD rat models hold for inflammatory markers as well. Our findings are in accordance with other investigations among elderly subjects whose diets lack n-3 fatty acid that results in a generation of an elevated level of pro-inflammatory markers [15, 22, 23]. The converse was true for n-3 fatty acids, which were associated with lower inflammation and serum cytokine levels [24].

CONCLUSION

The comparative assessment in three different rat models indicated that aging and hyperglycemia onset intensified the OS and inflammatory markers under dyslipidemic conditions. The restorative potential of dietary EPA + DHA was found to be superior when compared to dietary ALA in all three models assessed in this study. However, the molecular and epigenetic phenomena governing these changes in young and aged groups and also enzymatic aberrations incurred due to the onset of hyperglycemia under dyslipidemic conditions need to be further explored.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

Ethical Approval. All the experimental procedures performed in studies involving animals were in accordance with the ethical standards of CSIR-Central Food Technological Research Institute, Mysore, India. The experimental protocols involving animals were approved by the Institutional Animals Care and Use Committee (Approval Number IAEC No.311/14).

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