Olive Oil Decreases both Oxidative Stress and the Production of Arachidonic Acid Metabolites by the Prostaglandin G/H Synthase Pathway in Rat Macrophages[1,2]

Juan J. Moreno,* T. Carbonell,† T. Sánchez,* S. Miret† and Maria T. Mitjavila†[3]

*Department of Physiology, Faculty of Pharmacy and †Department of Physiology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain

ABSTRACT Fish oil has a preventive role in cardiovascular and inflammatory diseases, but little is known about the effect of olive oil, which is widely consumed in Mediterranean regions. We examined the influence of dietary olive oil, corn oil and fish oil-rich diets on the production of superoxide anion (O_2^-) and nitric oxide ($^{\cdot}NO$) by resident macrophages stimulated by phorbol 12-myristate 13-acetate (PMA) and their effect on arachidonic acid release, prostaglandin G/H synthase-2 (PGHS-2) expression and the subsequent prostaglandin E_2 production. Resident peritoneal macrophages stimulated by PMA from rats fed with olive oil or corn oil had the same level of O_2^- production, but these levels were increased by the fish oil diet. Olive oil and the fish oil diets increased $^{\cdot}NO$ and decreased arachidonic acid mobilization and the production of prostaglandin E_2. PGHS-2 expression, however, was not affected by diet. We conclude that although olive oil and fish oil reduce arachidonic acid mobilization and subsequent metabolism through the PGHS-2 pathway in PMA-stimulated macrophages, only olive oil offers an additional beneficial effect by increasing $^{\cdot}NO/O_2^-$ production. J. Nutr. 131: 2145–2149, 2001.

KEY WORDS: • *fish oil* • *nitric oxide* • *olive oil* • *prostaglandins* • *superoxide anion* • *rats*

A key factor in both the development and treatment of certain diseases is the type of fatty acid in the diet (1,2). Thus, Mediterranean populations show a low prevalence of coronary disease (3,4), despite consuming diets in which ~40% of the energy is derived from fat (5). It has been postulated that this apparent protection from coronary diseases may be due to the high proportion of dietary monounsaturated fatty acids relative to saturated fatty acids and the high doses of antioxidants consumed by these populations (6).

Fish oil also exerts beneficial effects on cardiovascular and chronic inflammatory diseases. Eicosapentaenoic acid and docosahexaenoic acid are the (n-3) PUFA responsible for these effects, because they reduce the synthesis of proinflammatory eicosanoids (7). A fish oil-rich diet also affects the generation of free radicals, including superoxide anion (O_2^-) and nitric oxide ($^{\cdot}NO$)[4] as we have demonstrated elsewhere in phagocytes (7). Moreover, the Mediterranean diet is also rich in fish.

Arachidonic acid (AA) is an important PUFA of cell membrane phospholipids and also a cellular mediator that acts by itself or following its transformation to eicosanoids, its oxidized biologically active products. Under physiological conditions, the amount of free intracellular AA available is quite small. However, AA release from phospholipids occurs through the activation of phospholipases, primarily phospholipase A_2 (3.1.1.4). Then, free AA can be metabolized via prostaglandin G/H synthase (PGHS) (1.14.99.1), lipoxygenase (EC 1.13.11.12) or cytochrome P_{450} monooxygenase (1.6.2.3) pathways.

PGHS is the rate-limiting enzyme in the conversion of AA to prostaglandins and other eicosanoids, such as prostacyclins and thromboxanes. PGHS-1 is constitutively present in several cells and tissues (8), whereas PGHS-2 is usually absent in resting cells, although its expression is greatly increased by serum, cytokines and mitogens (9,10). Moreover, PGHS-2 activity may be modified in the presence of oxidant hydroperoxides (11) and $^{\cdot}NO$ (12,13).

The detection of PGHS-2 isoform in inflammatory sites (14–16) indicates a role for this enzyme in the development of the inflammatory response through the production of prostaglandins. Furthermore, the recruitment of mononuclear cells to the inflammatory sites and their subsequent activation appears to be involved in AA release, PGHS-2 overexpression and prostaglandin production during the inflammatory process (17).

The objective of this study was to determine the effect of an olive oil-rich diet on oxidative stress and on prostaglandin production, important mediators of inflammation. Moreover, we compared olive oil with a corn oil-rich diet and with a fish

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[3] To whom correspondence should be addressed. E-mail: tmitja@bio.ub.es
[4] Abbreviations used: AA, arachidonic acid; BSA, bovine serum albumin; $^{\cdot}NO$, nitric oxide; O_2^- superoxide anion; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PGHS, prostaglandin G/H synthase; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.

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oil-rich diet, a well-documented diet recommended to prevent cardiovascular and inflammatory diseases.

MATERIALS AND METHODS

Reagents. RPMI 1640, heat-inactivated fetal calf serum, penicillin and streptomycin were purchased from Life Technologies (Gaithersburg, MD). [5,6,8,9,11,12,14,15-^{3}H]AA (180–240 Ci/mmol) was obtained from DuPont/New England Nuclear (Boston, MA). Aprotinin, leupeptin, diethyldithiocarbamic acid, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), olive oil, corn oil, fish (menhaden) oil, all-*rac*-α-tocopherol acetate, phorbol 12-myristate 13-acetate (PMA), superoxide dismutase (SOD) from bovine liver and cytochrome *c* from horse heart (type VI) were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antiserum directed against PGHS-2, sheep PGHS-2, prostaglandin E_2 (PGE_2)-monoclonal enzyme immunoassay kit, and *N*-imino-ethyl-L-ornithine were from Cayman Chemicals (Ann Arbor, MI). All other reagents were of analytical grade.

Animals and diets. After weaning, male Sprague-Dawley rats (Harlan Iberica, Barcelona, Spain) were randomly divided into three groups of six rats and fed for 16 wk with isoenergetic semipurified diets (**Table 1**). Diets contained 5 g/100 g lipids as olive oil, [rich in 19:1(n-9)], corn oil [rich in 18:2(n-6)] or fish (menhaden) oil [rich in 20:5(n-3) and 22–6(n-3)]. The α-tocopherol contained in the oils only provided between 2.3 and 2.5 mg/kg of each diet and diets were supplemented with 100 mg/kg all-*rac*-α-tocopherol acetate (67 IU α-tocopherol/kg). Food was provided daily and all leftovers were removed. Diets were manufactured weekly and stored at −20°C under vacuum to prevent oxidation. The fatty acid composition of the diets was determined according to Haan et al. (18) (**Table 2**). The experimental protocols were reviewed and approved by the Ethical Committee of the Faculty of Biology in accordance with European Community guidelines.

Macrophages. Resident peritoneal macrophages were collected from rats anesthetized with diethyl ether, and the peritoneal cavity was washed as described previously (19). Macrophages were counted microscopically using May Grunwald-Giemsa dye, and $15 \pm 3 \times 10^6$ macrophages were recovered per rat. The cell viability, which was measured by the Trypan blue exclusion test, was > 90% in the three dietetic groups. Macrophages were then purified by adherence to plastic culture plates (2 h at 37°C in 95% air, 5% CO_2).

Measurement of O_2^- and ˙NO production. The medium was removed and replaced by Hank's balanced salt solution without phenol red. The production of O_2^- in response to PMA was measured by the reduction of cytochrome *c* at 550 nm (20). Preliminary studies demonstrated that PMA induced an early production of O_2^- that reached a plateau after 2 h (data not shown). Thus, macrophages (0.5×10^6) were incubated in 0.5 mL of Hank's balanced salt solution for 2 h at 37°C in the presence of 1.5 mg cytochrome *c* and 0.15 μmol/L PMA or 150 IU SOD or both. The O_2^- concentration was calculated using the molar absorption coefficient of 21 $(mmol/L)^{-1} \cdot cm^{-1}$. The amount of O_2^- released by macrophages was calculated from the difference between the absorbance of the samples with and without SOD.

The production of ˙NO was measured as nitrite by the Griess reaction. Preliminary studies using macrophages showed that nitrite, the stable product of ˙NO with molecular oxygen, started to be detected at 18 h and reached a plateau after 24 h (data not shown). Macrophages (0.25×10^6) were incubated in 0.25 mL of RPMI 1640 medium without phenol red for 24 h at 37°C with 0.15 μmol/L PMA in the absence or presence of 0.5 mmol/L *N*-imino-ethyl-L-ornithine. The medium was removed and mixed with an equal volume of Griess reagent [0.5% *N*-(1-naphthyl)ethylenediamine, 0.5% sulfanilamide and 2.5% phosphoric acid]. Nitrite concentration was calculated by reference to a standard curve and expressed as nitrite released by macrophages.

Incorporation and release of [^{3}H]AA. Macrophages (10^6/well) were incubated in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics (1×10^5 U/L of penicillin and 100 mg/L of streptomycin) for 20 h. The RPMI 1640 medium was removed and replaced with 0.5 mL RPMI 1640 containing 0.1 μCi of [^{3}H]AA. Samples were incubated for 6 h at 37°C. Cells were then washed three times in medium containing 5 g/L BSA to remove unincorporated [^{3}H]AA. The labeled cells were incubated with 1 μmol/L PMA for 2 h. The medium was removed for analysis of the radioactivity released. At the end of each experiment, the cell monolayer was overlaid with 0.1% Triton X-100, and the cells were scraped off. The radioactivity present in the medium and in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter (Packard Bioscience Co., Downers Grove, IL). The spontaneous [^{3}H]AA release by nonstimulated cells representing each diet condition was similar and subtracted. The amount of [^{3}H]AA incorporated in terms of dpm was 15,263 ± 1,756, 14,756 ± 1,758 and 16,326 ± 1,568 for olive oil, corn oil and fish oil, respectively, which was determined in solubilized cells. The amount of [^{3}H]AA released into the medium was expressed as a percentage of cell-incorporated [^{3}H]AA.

PGHS-2 Western blot analysis. Macrophages were washed twice in ice-cold phosphate-buffered saline (PBS) solution and scraped off

TABLE 1

Composition of semipurified diets

Component	Amount
	g/kg diet
Casein[1]	220
Cornstarch	446
Sucrose	228
Cellulose	10
DL-Methionine	1
Mineral mix[2]	35
Vitamin mix[3]	10
Oil[4]	50

1 Vitamin free delipidated.
2 AIN-93M (ICN Pharmaceuticals, Costa Mesa, CA).
3 AIN-93VX (ICN Pharmaceuticals).
4 Olive oil, corn oil or fish (menhaden) oil.

TABLE 2

Fatty acid composition of olive oil, corn oil and fish oil semipurified diets

Fatty acids	Olive oil	Corn oil	Fish oil
		g/kg diet	
14:0	1.02	0.80	0.59
16:0	5.25	6.55	9.38
16:1	0.32	0.12	5.1
18:0	1.72	1.25	2.1
18:1(n-9)	36.25	12.15	6.25
18:1(n-7)	—[1]	—	0.55
18:2(n-6)	3.8	28.3	0.86
18:3(n-6)	—	—	0.14
18:3(n-3)	0.55	—	0.92
20:1(n-9)	—	—	0.81
20:2(n-6)	—	—	2.45
18:4(n-3)	0.09	—	0.12
20:4(n-6)	0.18	—	0.70
22:1(n-9)	—	—	0.12
20:5(n-3)	—	0.06	8.73
22:4(n-6)	—	0.08	0.01
24:1(n-9)	—	—	0.34
22:5(n-6)	—	—	0.20
22:5(n-3)	—	0.09	0.02
22:6(n-3)	—	0.11	4.27

1 Indicates that <0.5 g/kg of the fatty acid was present or that it was not detected.

in PBS containing 2 mmol/L EDTA and pelleted. Cell pellets were sonicated in PBS containing 2 mmol/L EDTA, 20 mg/L phenylmethylsulfonyl fluoride, 20 mg/L aprotinin, 20 mg/L leupeptin and 200 mg/L diethyldithiocarbamic acid. Total protein was measured by the Bradford method by means of the Bio-Rad (Hercules, CA) detergent-compatible protein assay, using BSA as standard.

Cell lysates (~20 μg protein) were separated by 10% SDS-PAGE and blotted for 1 h with a constant current of 250 mA onto a nitrocellulose membrane (*trans*-blot, 0.4-μm pore size) using a Miniprotean II system (Bio-Rad). Sheep PGHS-2 purified from placenta was also loaded on the gels as positive control. A prestained SDS-PAGE protein standard (Bio-Rad) was used to check transfer efficiency. The membranes were blocked with 50 g/L nonfat milk powder in PBS 0.1% Tween 20 for 1 h. A rabbit polyclonal antiserum directed against PGHS-2 (synthetic peptide from murine PGHS-2) was applied at a dilution of 1:2,000 for 1 h. The blot was washed several times in PBS Tween 20 and incubated with a goat anti-rabbit antibody at a dilution of 1:2,000 for 1 h. Antibody binding was visualized by the enhanced chemiluminescence technique, according to the instructions of the supplier, using Kodak X-OMAT LS film (Rochester, NY).

Measurement of PGE_2 production. An aliquot (0.25 mL) of the supernatant culture medium was acidified with 1 mL of 1% formic acid. PGE_2 was extracted in ethyl acetate (5 mL) and the organic phase was evaporated in a stream of nitrogen. The overall recovery established by addition of [^{3}H]PGE_2 was 80%. PGE_2 levels in the medium were determined by electroimmunoassay using a PGE_2-monoclonal enzyme immunoassay kit (Cayman) following the protocol of the manufacturer.

Statistical methods. Statistical analysis of the data was performed using GraphPad InStat Statistical software, Version 2.04 (San Diego, CA). Results are expressed as means ± SEM. Data were evaluated using either the unpaired Student's *t* test or one-way ANOVA. Homogeneity of variances was tested by the Barlett's test. The Student-Newman-Keuls multiple comparison test was used to detect differences among groups ($P < 0.05$).

RESULTS

Food consumption and animal growth. No significant differences were observed in daily food consumption among the groups. Consequently, the time course of the weight increase shown by each group of rats was similar. Final body weights were 441 ± 36, 435 ± 30 and 401 ± 35 g for rats fed the olive oil, corn oil and fish oil diets, respectively.

Effect of diets on O_2^- and $^{\cdot}NO$ production. The increase in production of O_2^- by peritoneal macrophages stimulated with 1 μM PMA was similar in cells from rats fed olive (5.87 ± 2.99 nmol) and corn oil (7.52 ± 1.43 nmol) (**Fig. 1**) and significantly higher (16.02 ± 5.90 nmol) in fish oil-fed rats.

The lowest nitrite production after stimulating cells with PMA was observed in the peritoneal macrophages of rats fed the corn oil diet (71.3 ± 15.7 nmol). Production was significantly higher in rats fed olive (75%) and fish (78%) oil diets (**Fig. 2**).

The highest nitrite/O_2^- production ratio was observed in cells from rats fed the olive oil diet (data not shown).

Effect of diets on [^{3}H]AA release, PGHS-2 expression and PGE_2 synthesis. The amount of free intracellular AA available is quite small in nonstimulated cell cultures. However, the release of [^{3}H]AA from cellular phospholipids after macrophage activation is an early response to several stimuli. Our results show that the release of [^{3}H]AA by resident peritoneal macrophages stimulated by 1 μmol/L PMA for 2 h was lower in rats fed olive (14.3 ± 1.6%) or fish oil (19.7 ± 2.3%) diets than it was in cells from rats fed the corn oil (28.3 ± 3.6%) diet (**Fig. 3**), although all cells incorporated [^{3}H]AA to a similar extent (48 ± 3% in rats fed the corn oil diet).

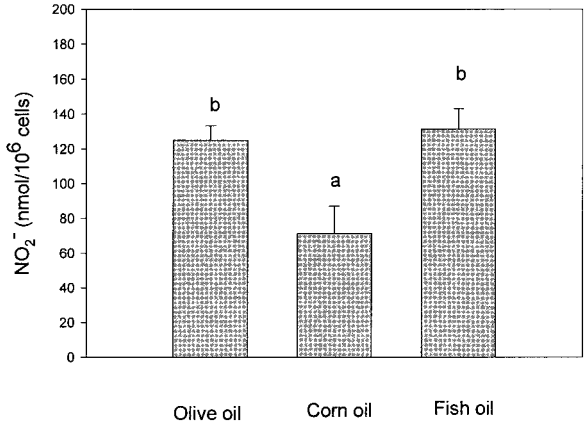

FIGURE 2 $^{\cdot}NO$ production by resident peritoneal macrophages from rats fed olive, corn or fish oil diets for 16 wk. Cells/well (0.25×10^6) were incubated in 0.25 mL PBS with 0.15 μmol/L PMA for 2 h. Values are the mean ± SEM, n = two replicates from four to five separate experiments. Means with different letters differ, $P < 0.05$.

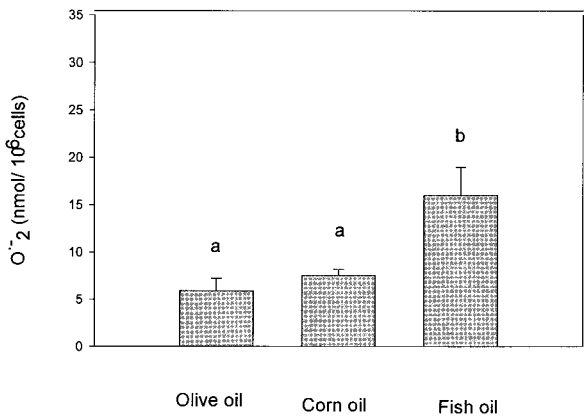

FIGURE 1 O_2^- production by resident peritoneal macrophages from rats fed olive, corn or fish oil diets for 16 wk. Cells/well (0.5×10^6) were incubated in 0.5 mL PBS with 0.15 μmol/L PMA for 2 h. Values are means ± SEM, n = two replicates from four to five separate experiments. Means with different letters differ, $P < 0.05$.

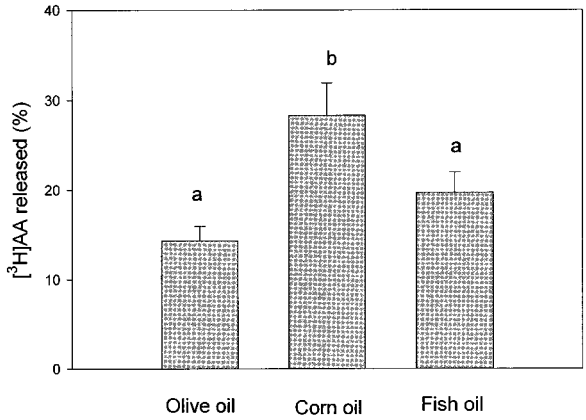

FIGURE 3 [^{3}H]AA release by resident peritoneal macrophages from rats fed olive, corn or fish oil diets for 16 wk. Cells/well (10^6) were stimulated with 1 μmol/L PMA for 2 h. Values are the mean ± SEM, n = two replicates from four to five separate experiments. Means with different letters differ, $P < 0.05$.

To examine the expression of PGHS-2 in PMA-stimulated peritoneal macrophages, we performed immunoblot analyses using a specific antibody against PGHS-2. PGHS-2 was barely detectable in nonstimulated cells from rats feds all three diets. However, the PGHS-2 levels expressed by macrophages incubated with 1 μmol/L PMA for 2 h increased markedly (~15-fold) (**Fig. 4**). We did not observe appreciable differences in PGHS-2 levels induced by PMA in macrophages obtained from rats in the three groups (Fig. 4). In contrast, diet influenced the production of PGE_2 by macrophages stimulated by PMA, and maximal PGE_2 levels were observed in the cells of rats fed the corn oil diet, whereas cells from rats fed the olive and the fish oil diets produced 31% and 51% less PGE_2, respectively (**Table 3**). These differences in PGE_2 synthesis by macrophages from the three groups might be a consequence of differences in the capacity to release AA as we observed above.

DISCUSSION

The main source of fat in the Mediterranean diet is olive oil. The composition of this oil differs from that of other vegetable oils (corn or sunflower) that are currently consumed in many countries. Olive oil contains a high amount of oleic acid and only a small amount of linoleic acid, whereas corn oil contains a high amount of the latter, the precursor of AA in mammals (Table 2). Moreover, olive oil promotes the inclusion of docosahexaenoic acid and the exclusion of (n-6) docosapentaenoic acid. Olive oil, by inhibiting the Δ6 desaturase, reduces the conversion of linoleic acid to AA (21–23) and, thus, presents anti-inflammatory activity. Furthermore, olive and corn oils contain different amounts and types of plant sterols and other substances, such as polyphenols and vitamins.

The (n-3) PUFA contained in fish oil provide it with anti-inflammatory effects (24,25). Although the mechanism is not clear, it appears that the fatty acid composition of cell membranes is determined by the fatty acid content of the diet (26). Bartoli et al. (27) recently reported that an olive oil diet significantly reduced AA concentration in tissues. These changes might be responsible for the impairment of the synthesis of AA metabolites involved in inflammation, such as PGE_2. Similarly, we recently observed that a fish oil diet induced changes in AA levels in biological membranes and a significant impairment in [^{3}H]AA mobilization when macrophages were stimulated with proinflammatory agents, such as PMA, calcium ionophore A23187 or opsonized zymosan (28). The present study was designed to examine the effects of an olive oil-rich diet on O_2^- and $^{\cdot}NO$ production as well as its effects on AA mobilization, PGHS-2 expression and the subsequent prostaglandin generation by resident peritoneal macrophages stimulated ex vivo by PMA and to compare the results with those from fish oil- and corn oil-rich diets.

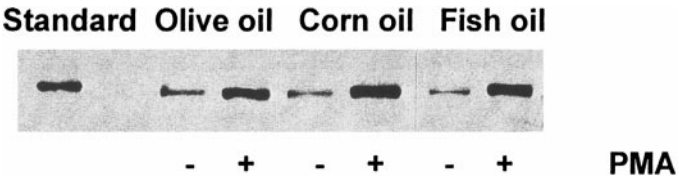

FIGURE 4 Western blot analysis of PGHS-2 in peritoneal macrophages from rats fed olive, corn or fish oil diets for 16 wk stimulated with 1 μmol/L PMA for 2 h. The Western blot is representative of three experiments.

TABLE 3

Effect of PMA on PGE_2 synthesized by macrophages isolated from rats fed olive, corn or fish oil diets for 16 wk[1,2]

	PGE_2		
	Olive oil	Corn oil	Fish oil
	μg/L		
Control	12.1 ± 0.6	15.2 ± 0.7	12.4 ± 1.0
PMA	172 ± 8[b*]	248 ± 7[c*]	121 ± 5[a*]

[1] Values are means ± SEM, n = two replicates from three rats.
[2] Macrophages (10^6) were incubated with 1 mM PMA for 2 h.
* Different from control, $P < 0.001$. Means in a row with different letters differ, $P < 0.05$.

Our results show that PMA-induced O_2^- and $^{\cdot}NO$ production by macrophages from rats fed a fish oil-rich diet were higher than that produced from rats fed a corn oil-rich diet, whereas the olive oil-rich diet only increased $^{\cdot}NO$ production. $^{\cdot}NO$ has been described as prooxidant by the generation of peroxynitrite ($ONOO^-$) in the presence of O_2^- or as an antioxidant (29). It is likely that under the conditions of the present study, the level of O_2^- reached in the vicinity of macrophages from rats fed the fish oil diet was higher than that in the macrophages from rats fed the olive oil diet. These data provide additional evidence that olive oil is more efficient in reducing oxidative stress than fish oil. These beneficial effects have been attributed, at least in part, to the phenolic compounds of olive oil, such as oleuropein and hydroxytyrosol, which inhibits the PMA-elicited respiratory burst of human polymorphonuclear cells (30). Moreover, olive oil provides monounsaturated fatty acids, which are not as readily oxidizable as the PUFA provided by fish oil (31,32). Thus, an olive oil-rich diet might reduce membrane susceptibility to lipid peroxidation more than a fish oil-rich diet. This hypothesis is supported by recent studies, which demonstrate that the ingestion of olive oil increases resistance to lipid peroxidation (33–35).

Furthermore, PGE_2 levels induced by PMA in the culture medium of macrophages from rats fed the olive oil and fish oil diet were significantly lower than those from rats fed the corn oil diet, as was also observed by Bartoli et al. (27). PGHS-2 is involved in PGE_2 synthesis when resident peritoneal macrophages are stimulated by PMA. The present study shows that the PGHS-2 overexpression in macrophages stimulated by PMA was not affected by the three diets. Although our results differ from those reported by Lo et al. (36), the discrepancy can be attributed to the fact that they used RAW 264.7 macrophages incubated in an eicosapentaenoic acid-rich medium. Macrophages isolated from rats fed olive oil or fish oil-rich diets released less [^{3}H]AA when they were stimulated by PMA. Thus, the impairment of PGE_2 synthesis observed in rats fed olive oil or fish oil diets may be associated with changes in the fatty acid composition of the phospholipids bilayer, specifically the reduction in AA levels and AA mobilization (22,28,36). However, our results can be explained not only by changes in the absolute or relative levels of specific eicosanoid precursors, but also by general effects on membrane composition, structure and function induced by fatty acids. The nitrite/O_2^- ratio produced by cells from rats fed the olive oil diet may affect PGHS activity and consequently PGE_2 synthesis, as proposed by other groups (11–13). Moreover,

unsaponifiable fractions of olive oil may also modulate eicosanoids synthesis (37) and anti-inflammatory effects (38).

Several authors have suggested that O_2^- and/or $^{\cdot}NO$ might be involved in the signal transduction cascade that controls AA release and the subsequent metabolism by the PGHS-2 pathway (13,39,40). However, the O_2^- and $^{\cdot}NO$ generated by PMA-stimulated macrophages were not correlated with the differences in PGE_2 synthesis due to olive, corn or fish oil diets under our experimental conditions.

In conclusion, our findings indicate that an olive oil diet, like with a fish oil diet, reduces AA release and subsequent production of AA metabolites by the PGHS-2 pathway, which is induced by PMA in resident peritoneal macrophages. However, PMA induced less oxidative stress in macrophages from rats fed the olive oil diet than from those fed the fish oil diet, by increasing the $^{\cdot}NO/O_2^-$ ratio.

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