

Inhibition by Interferon- γ of Human Mononuclear Cell-mediated Low Density Lipoprotein Oxidation

Participation of Tryptophan Metabolism along the Kynurenine Pathway

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Abstract

In this study we examined the potential inhibition by interferon- γ (IFN γ) of the early stages of low density lipoprotein (LDL) oxidation mediated by human peripheral blood mononuclear cells (PBMC) and monocyte-derived macrophages (MDM) in Ham's F-10 medium supplemented with physiological amounts of L-tryptophan (Trp). We assessed LDL oxidation by measuring the consumption of LDL's major antioxidant (i.e., α -tocopherol) and targets for oxidation (cholesteryllinoleate and cholesteryl arachidonate), together with the accumulation of cholesteryl ester hydroperoxides and the increase in relative electrophoretic mobility of the lipoprotein particle. Exposure of PBMC or MDM to IFN γ induced the degradation of extracellular Trp with concomitant accumulation of kynurenine, anthranilic and 3-hydroxyanthranilic acid (3HAA) in the culture medium. Formation of 3HAA, but neither Trp degradation nor formation of kynurenine and anthranilic acid, was inhibited by low amounts of diphenylene iodonium (DPI) in a concentration-dependent manner. In contrast to oxidative Trp metabolism, exposure of human PBMC or MDM to IFN γ failed to induce degradation of arginine, and nitrite was not detected in the cell supernatant, indicating that nitric oxide synthase was not induced under these conditions. Incubation of LDL in Trp-supplemented F-10 medium resulted in a time-dependent oxidation of the lipoprotein that was accelerated in the presence of PBMC or MDM but inhibited strongly in the presence of both cells and IFN γ , i.e., when Trp degradation and formation of 3HAA were induced. In contrast, when IFN γ was added to PBMC or MDM in F-10 medium that was virtually devoid of Trp, inhibition of cell-accelerated LDL oxidation was not observed. Exogenous 3HAA added to PBMC or purified monocytes in the absence of IFN γ also strongly and in a concentration-dependent manner inhibited LDL oxidation. Selective inhibition of IFN γ -induced formation of 3HAA by DPI caused reversion of the inhibitory action of this cytokine on both PBMC- and MDM-mediated LDL oxidation. These results show that IFN γ treatment of human PBMC or MDM in vitro attenuates the extent of LDL oxidation caused by these cells,

and indicate that Trp degradation with formation of 3HAA is a major contributing factor to this inhibitory activity. (*J. Clin. Invest.* 1994. 93:2149–2158.) Key words: atherosclerosis • 3-hydroxyanthranilic acid • indoleamine 2,3-dioxygenase • lipid hydroperoxide • macrophages

Introduction

Oxidative modification of LDL may be important in the in vivo formation of lipid-laden cells (i.e., foam cells) that significantly contribute to the development of atherosclerosis (1, 2). Although the precise molecular mechanism(s) underlying oxidative LDL modification in vivo remains unknown, in vitro studies on cell-mediated LDL oxidation are generally carried out in medium containing small amounts of the transition metals iron and/or copper. Under these conditions all cell types present in a lesion (i.e., endothelial cells [3], macrophages [4], smooth muscle cells [5, 6] and lymphocytes [7]) are capable of oxidizing LDL. Where investigated directly, in vitro cell-mediated LDL oxidation has been shown to have an absolute requirement for the presence of iron and copper in the medium (3, 6); i.e., in their absence, these cells do not oxidize LDL lipids substantially. The resulting modified LDL can be taken up readily by macrophages, turning them into foam cells similar to those observed in atherosclerotic lesions in vivo (8). Antioxidants such as probucol (9), ascorbate (10), or ubiquinol-10 (11), when added to these and other systems, suppress or completely inhibit LDL oxidation, indicated by the decrease in the lipid hydroperoxides formed or the preservation of LDL-associated α -tocopherol (α -TOH).¹

Inhibition of murine macrophage-mediated LDL oxidation has also been observed by treatment of the cells with interferon- γ (IFN γ) (12–14). IFN γ -induced attenuation of cell-mediated LDL oxidation correlated with induction of nitric oxide radical (NO) synthesis from arginine (12, 14). In addition to NO formation, IFN γ can induce at least two other pathways in mononuclear phagocytes that may attenuate LDL oxidation: oxidative degradation of heme and Trp initiated by heme oxygenase and indoleamine 2,3-dioxygenase (IDO), respectively. Both pathways eliminate potential pro-oxidants while producing powerful antioxidants (15–19) (Scheme I).

We are interested in oxidative Trp degradation along the kynurenine (Kyn) pathway and the concomitant formation of

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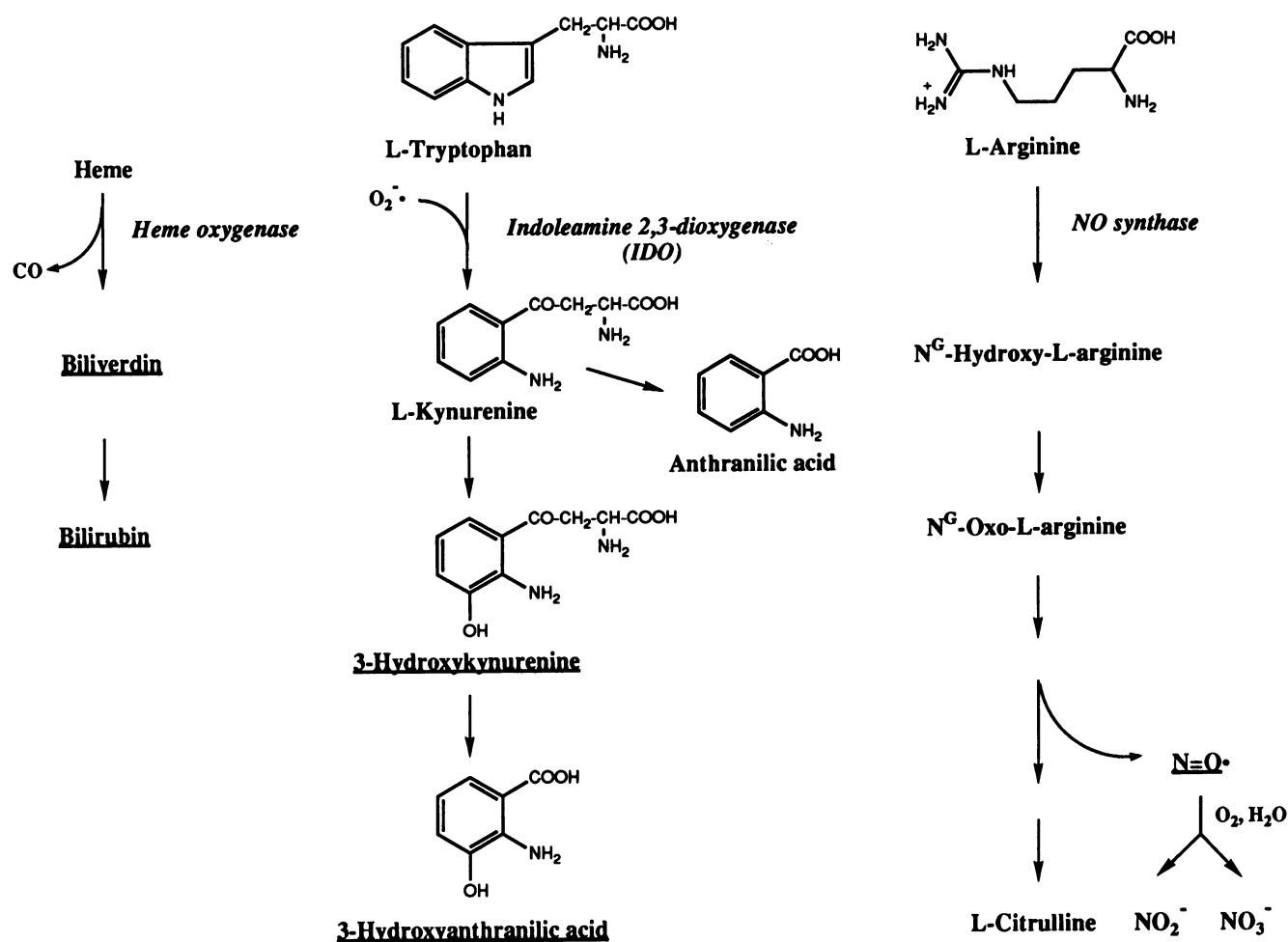
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1. Abbreviations used in this paper: CE-OOH, cholesteryl ester hydroperoxides; C18:2, cholesteryllinoleate; C20:4, cholesteryl arachidonate; DPI, diphenylene iodonium; 3HAA, 3-hydroxyanthranilic acid; IDO, indoleamine 2,3-dioxygenase; Kyn, kynurenine; NO, nitric oxide radical; MDM, monocyte-derived macrophages; α -TOH, α -tocopherol; Trp, L-tryptophan.



Scheme 1. Interferon- γ -mediated induction in mononuclear phagocytes of pathways that may provide additional antioxidant protection. Metabolites with reported antioxidant activity are underlined.

the antioxidant 3-hydroxyanthranilic acid (3HAA) because this pathway is readily induced by IFN γ in human (20, 21) monocytes/macrophages but not murine monocytes/macrophages (22) or human lymphocytes. In contrast, IFN γ (together with LPS) readily induces NO formation in murine macrophages while at least in vitro this pathway is not induced in human monocytes/macrophages by this and other cytokines. Because a large proportion of lymphocytes present in atherosclerotic lesions appear to be activated, local release of IFN γ could result (23–25). We therefore examined whether IFN γ attenuated LDL oxidation mediated by human PBMC or monocyte-derived macrophages (MDM) and, if so to what extent oxidative degradation of arginine and/or Trp contributed to this activity. Oxidative modification of LDL was assessed by measuring the consumption of both α -TOH and polyunsaturated esterified lipids, as well as formation of cholesteryl ester hydroperoxides (CE-OOH) and changes in net surface charge of the lipoprotein. Induction of IDO and NO synthase (Scheme 1) was determined by measuring Trp metabolites and nitrite, respectively, in the cell culture supernatants. The results obtained indicate that induction of IDO but not NO synthase participates in the observed inhibition by IFN γ of human PBMC- or MDM-mediated LDL oxidation.

Methods

Materials. 3HAA, anthranilic acid, Kyn, EDTA, PBS, HBSS, (both Ca^{2+} - and Mg^{2+} -free) and RPMI 1640 (powder) were obtained from Sigma Chemical Co. (St. Louis, MO). L-Trp, chloroacetic and trichloroacetic acid (TCA) were from Merck (Darmstadt, FRG), Ham's F-10 medium (control No. 19K6422) from Gibco (Grand Island, NY), human recombinant IFN γ and chloramphenicol from Boehringer-Mannheim GmbH (Mannheim, FRG), and potassium bromide from British Drug House (BDH, Poole, England). Diphenylene iodonium (DPI) was generously provided by Prof. O.T.G. Jones (University of Bristol, UK) and given to us as a gift by Dr. W. Jessup (Heart Research Institute); it was stored at $-20^\circ C$ as a 1 mM stock solution in 50% ethanol (vol/vol). *Tert*-butyl alcohol and ethanol were from Rhône-Poulenc (Paris, France) and BDH, respectively (both analytical grade), and all other organic solvents (HPLC quality) from Mallinckrodt, Inc. (Chesterfield, MO). LiquiPure water (MODULAB) was used for buffers and aqueous solutions, which were subsequently treated with Chelex-100 (Bio-Rad Laboratories, Richmond, CA) to remove contaminating transition metals. Buffers and media used for cell isolation, elutriation and culture (except for F-10 media) were sterile-filtered through Zetapor membranes (CUNO, Meriden, CT) and stored in heat-treated ($250^\circ C$ for 3 h) glassware to minimize contamination with endotoxin (LPS), tested for regularly using a chromogenic *Limulus* amoebocyte lysate test (26) (Associates of Cape Cod/American Diagnostica, Greenwich, CT) and found to be < 50 pg/ml.

Preparation of LDL. Human LDL ($d \approx 1.06$ g/ml) was isolated from anticoagulated (EDTA-K₃ vacutainers, Becton Dickinson & Co., Mountain View, CA) plasma obtained from nonfasted, healthy, and normolipidemic donors by rapid ultracentrifugation (2 h, 15°C) (TL-100.4 rotor in a TL-100 benchtop centrifuge, Beckman Instruments, Inc., Fullerton, CA) as described (27). The isolated LDL was then dialyzed for 18 h against four changes of 100 vol of deoxygenated 10 mM PBS, pH 7.0, containing 0.1 mg/l chloramphenicol and 1 g/l Chelex-100. Chelex was omitted in the last dialysis step. For some experiments, the isolated LDL was gel-filtered (PD-10 column, Pharmacia) rather than dialyzed. In all cases, the LDL was then filter sterilized (0.45 μ m) and used immediately in experiments. LDL prepared in this way consistently contained only low levels of cholesteryl ester hydroperoxides (CE-OOH), i.e., 45 ± 38 pmol CE-OOH/mg LDL protein (mean \pm SD, $n = 10$), corresponding to ≈ 1 CE-OOH molecule for every 43 LDL particles. LDL protein concentration was determined with BSA as the standard and using either the bicinchoninic acid method (Sigma Chemical Co.) or the modified procedure of Peterson (28) (Sigma Kit P 5656).

Isolation of cells. Where feasible, PBMC were isolated from blood obtained from the same donor used for the LDL preparation. Blood was obtained under the guidelines and approval of the local human ethics committee. For isolation of large numbers of PBMC, monocytes or MDM, white blood cell concentrates ("buffy coats," kindly provided by the N.S.W. Red Cross Blood Transfusion Service, Sydney) were used. PBMC were isolated from diluted plasma or buffy coats by centrifugation on Lymphoprep (NYCOMED, Oslo, Norway) and cultured under nonadherent conditions (29) at 5×10^6 cells/ml in serum-free Ham's F-10 medium containing either 10% (vol/vol) heat-inactivated FCS (MultiSer, Cytosystems, Castle Hill, Australia) or 15% (vol/vol) human AB serum (Red Cross).

Human monocytes (used at 1×10^6 cells/ml) and lymphocytes were purified from PBMC (prepared from buffy coat) using counterflow centrifugal elutriation (30) carried out at 21°C. The elutriation system consisted of a Beckman JE-6 rotor in a Beckman J2-21M/E centrifuge equipped with strobe assembly. Flow through the system was controlled with a model 7545 Masterflex pump (Cole-Parmer Instrument Co., Chicago, IL) equipped with a 7021-20 head. PBMC, washed once with elutriation medium, were loaded into the elutriation chamber in a final volume of 50 ml ($\approx 1 \times 10^9$ cells) at a rotor speed of 2,020 rpm and an initial flow rate of 9.0 ml/min that was then increased by 0.5 ml every 10 min. Platelets eluted immediately, followed by lymphocytes (at a flow rate of ≈ 11.0 to 12.5 ml/min) and a mixed lymphocyte/monocyte fraction (up to ≈ 15 ml/min), and finally purified monocytes (at ≈ 15.0 ml/min). After complete elution of this mixed cell population, the flow rate was increased to 40 ml/min to elute the remaining purified monocyte fraction. This procedure resulted in lymphocyte (> 99% purity) and monocyte preparations (> 95% purity, as judged by nonspecific esterase staining, Sigma Kit 91-A) of > 99% viability (trypan blue exclusion).

For MDM preparations, elutriated monocytes were washed and resuspended in RPMI-1640, and adhered for 90 min at 37°C in 22-mm diam tissue culture wells (Costar, Cambridge, MA) at a concentration of $1-3 \times 10^6$ cells/well. After adherence, the medium was replaced with 1.5 ml RPMI-1640 with 10% (vol/vol) heat-inactivated human serum, 20 mM glutamine, 100 IU/ml penicillin and 100 μ g/ml streptomycin, and the cells left to differentiate for 6 d at 37°C in 5% CO₂ in air with media changes on days 3 and 5. On day 6, MDM were > 99% esterase positive and routinely yielded ≈ 200 μ g protein/ 10^6 cells.

Oxidative modification of LDL. Ham's F-10 medium was used for all experiments; the medium contains ≈ 3 μ M Trp and unless stated otherwise, was supplemented with 75–100 μ M L-Trp, a concentration similar to that in human plasma, i.e., 60 ± 15 μ M (31). For PBMC and purified monocytes experiment, LDL (≈ 2.5 mg protein/ml) (1 vol) was incubated in Ham's F-10 medium (10 vol) for up to 24 h at 37°C in a humidified atmosphere (5% CO₂ in air) in the presence or absence of cells. Aliquots (300 μ l) of cell-free medium or cell suspension were

withdrawn at various times and, where needed, cells removed by centrifugation (400 g for 10 min at 4°C). For MDM experiments, 0.6 ml of LDL (100 μ g/ml F-10 medium) was added per well and incubated for 24 h before the supernatant (600 μ l) was removed for lipid and antioxidant analyses. Where indicated, human IFN γ was added at 1,000 U/ml. In the case of MDM, cells were preincubated for 20 h with 100 or 250 U/ml of IFN γ before addition of LDL. The relative electrophoretic mobility of oxidized LDL ($\approx 1-2$ μ g) was determined using precast agarose gels (Ciba-Corning, Palo Alto, CA) subjected to electrophoresis in barbitone buffer (pH 8.6) at 90 V for 45 min and stained with Fat Red 7B stain (32). Before electrophoresis, the LDL contained in the culture medium was concentrated by centrifuge-assisted ultrafiltration (Lida, Kenosha, WI).

Determination of α -TOH, lipids, and CE-OOH by HPLC analysis. Lipid-soluble antioxidants (including α -TOH), neutral lipids (mainly free cholesterol and cholesteryl esters) and CE-OOH were extracted quantitatively from the LDL-containing cell culture supernatant as described (10, 27, 33, 34), and the extracts were stored at -70°C for up to 24 h before analysis. Where indicated, cholesterylbenzoate was added as an internal standard to the organic solvent before extraction (27). In all other cases, lipid-soluble components of LDL were standardized internally against cholesterol. The hexane extracts were dried under vacuum and redissolved in ethanol (180 μ l) for analyses by various HPLC methods. α -TOH and other lipid-soluble antioxidants, including ubiquinol-10, were determined by reversed-phase HPLC with electrochemical detection (10). In some experiments, α -TOH was determined in the lipid HPLC assay by fluorescence detection (292/330 nm) (Spectra Physics, San Jose, CA; FL2000). Unoxidized neutral lipids and CE-OOH were separated on an LC-18 column (Supelco Inc., Bellefonte, PA; 25×0.46 cm with 5-cm guard column, 5- μ m particle size), eluted with *tert*-butyl alcohol/methanol (1:1, vol/vol) at 1 ml/min and detected by ultraviolet absorbance at 210 nm and post-column chemiluminescence respectively, as described originally (33), with the modification described (27).

Assessment of Trp and arginine metabolism. For Trp metabolism, the culture medium (≈ 1 ml) was deproteinized with TCA (4% final concentration, wt/vol) after cells had been removed by centrifugation. Trp, Kyn, and anthranilic acid in the resulting supernatant were separated on a VeloSep RP-18 column (Applied Biosystems, Inc., Foster City, CA; 10×0.32 cm with 1-cm guard column, 3- μ m particle size) with 100 mM chloroacetic acid/acetonitrile (pH 2.2) (8:2, vol/vol) and detected photometrically (Kyn, 365 nm; Trp, 280 nm) or fluorimetrically (anthranilic acid, 328/422 nm) (35). 3HAA was determined by electrochemical detection (+0.5 V), either on-line with the above HPLC system (using an LKB/Pharmacia model 2143 detector with glassy carbon electrode) or after extraction into ethyl acetate and separation on an LC-18-DB column (Supelco, 25×0.46 cm with guard column, 5- μ m particle size) (29). Degradation of arginine was assessed by measuring the nitrite present in the supernatant of the LDL-modifying cell cultures using the Griess reagent (36); and/or by HPLC amino acid analysis using the *o*-phthalaldehyde method (37).

Statistical data analysis. The paired Student *t* test (one-tailed) was used to evaluate differences in the absolute values of the various groups of treatments. Significance was accepted at the $P < 0.05$ level, unless stated otherwise. Owing to the relatively large variations in antioxidant and lipid content of LDL preparations from different donors (38), α -TOH, and cholesteryl esters containing polyunsaturated fatty acids, i.e., cholesteryllinoleate (C18:2) and cholesteryl arachidonate (C20:4), were expressed as percent values relative to the initial concentration and presented as mean \pm SD or SE, and range. CE-OOH values were given in absolute concentrations.

Results

As has been reported by others (20, 39), incubation of PBMC or MDM in RPMI 1640 + 10% FCS and in the presence of

IFN γ resulted in induction of IDO as assessed by the time-dependent decrease of Trp and concomitant accumulation of Kyn, anthranilic acid, and 3HAA in the culture medium (not shown). In the absence of IFN γ , degradation of Trp and formation of Kyn pathway metabolites were much smaller, and exposure of purified lymphocytes to IFN γ in the absence of monocytes did not result in significant Trp degradation (not shown). These results demonstrate that IFN γ induces Trp degradation in human monocytes/macrophages but not lymphocytes. Furthermore, the presence of lymphocytes does not affect the extent of IFN γ -induced Trp degradation by monocytes (not shown, 39).

Studies on "cell-mediated" oxidative LDL modification are mostly carried out in serum-free Ham's F-10 (e.g., 3, 6), a medium that contains added transition metals. Where investigated directly, the transition metals have been demonstrated to be required for the oxidation process to proceed (3, 6). In studies using transition metal-poor RPMI 1640 medium, Cathcart and co-workers have shown that activated but not unactivated monocytes oxidize LDL as assessed by the comparatively less specific (to the methods used here) thiobarbituric acid reactive substances assay (e.g., 4, 40). To study a potential effect of Trp metabolism along the Kyn pathway on monocyte/macrophage-mediated LDL oxidation, we therefore initially examined the efficacy with which IFN γ induces oxidative Trp metabolism in PBMC cultured in Ham's F-10, a medium permissive for cell-mediated LDL oxidation. Table I shows that IFN γ also induced Trp degradation and metabolite formation in F-10 medium, with 3HAA and Kyn plus anthranilic acid accounting for ≈ 2 and 63% of the Trp lost, respectively. Although detected, 3-hydroxykynurenine, a precursor of 3HAA, did not accumulate to concentrations $> 0.2 \mu\text{M}$ (not shown). It has been reported recently (41) that quinolinic acid (which we did not measure and which does not contain antioxidant moieties) accounts for a significant proportion of the additional Trp degraded by human MDM; (see, however, reference 20). Addition of LDL to PBMC cultured in F-10 did not affect the extent of IFN γ -induced Trp degradation and metabolite formation (Table I). Cell viability (measured by trypan blue exclusion) in the (serum-free) F-10 medium was $\geq 95\%$ after 24 h of incubation and therefore did not affect our experiments on LDL oxidation (see below) which were carried out with cells cultured for up to 24 h only.

Incubation of PBMC or MDM in F-10 medium in the absence or presence of IFN γ for 24 h did not result in detectable

formation ($\geq 0.5 \text{ nmol}$) of extracellular nitrite (not shown). Treatment of these cells with IFN γ also did not result in a decrease in extracellular arginine, as assessed by amino acid analysis of the cell supernatant (not shown). These results indicate that the nitric oxide pathway was not induced under our conditions, consistent with data reported by others for human MDM (42), but in contrast to the situation observed with murine macrophages (see reference 12 and references cited therein).

Table II summarizes the results obtained for LDL oxidation in F-10 medium in the absence or presence of human PBMC or purified monocytes, and the effects of added IFN γ ($\approx 10^3 \text{ U/ml}$) or 3HAA ($10 \mu\text{M}$) on it. LDL oxidation was assessed by three different methods: the loss of α -TOH, quantitatively the major lipid-soluble antioxidant associated with LDL (38); the accumulation of CE-OOH, the major initial lipid oxidation product formed in oxidizing LDL (10, 34, 43); and the increase in LDL's relative electrophoretic mobility, an index of the surface charge of the LDL particle. As can be seen, incubation of LDL in Ham's F-10 medium for 24 h in the absence of cells resulted in oxidation of the lipoprotein, as indicated by alteration of all three oxidation parameters. Significant LDL lipid peroxidation was observed in the presence of α -TOH; in fact, on average some 36 molecules of CE-OOH were formed for each molecule of α -TOH consumed (Table II). Since the rates of α -TOH consumption and CE-OOH formation are indicative for the rates of initiation and propagation of lipid peroxidation in LDL respectively, these results demonstrate that cell-free LDL peroxidation in F-10 medium proceeded via a chain reaction, despite the presence of α -TOH. This is consistent with recent findings (44, 45) that indicated that under these conditions LDL lipid peroxidation is actually mediated by α -TOH. Cell-free LDL peroxidation was inhibited almost completely by the addition of $10 \mu\text{M}$ 3HAA.

Incubation of LDL in the presence of either PBMC or purified monocytes markedly accelerated lipoprotein oxidation, as indicated by a greater loss of α -TOH and extent of CE-OOH accumulation as well as increase in relative electrophoretic mobility (Table II). As was the case in the cell-free system, LDL lipid peroxidation occurred despite the presence of significant levels of α -TOH. On a molar basis, relatively more CE-OOH were detected than α -TOH lost. The simplest explanation for this is that cell-mediated LDL lipid peroxidation also proceeded via a chain reaction. Co-incubation of PBMC with IFN γ significantly decreased the extent of LDL oxidation (Ta-

Table I. Effects of IFN γ on Extracellular Levels of Trp and Kyn Pathway Metabolites Produced by Human PBMC Incubated in F-10 Medium in the Absence (–) or Presence (+) of LDL for 24 h at 37°C

Metabolite*	Trp		Kyn		AA		3HAA	
	–	+	–	+	–	+	–	+
LDL†								
Cell-free (μM)	95.8 \pm 5.7	102.5 \pm 7.3	0.28 \pm 0.4	0.40 \pm 0.3	ND	ND	<0.01	0.02 \pm 0.04
PBMC (nmol)	105.9 \pm 13.2	104.1 \pm 10.2	0.69 \pm 0.5	0.56 \pm 0.3	ND	ND	0.07 \pm 0.1	0.03 \pm 0.04
PBMC/rIFN γ ($\approx 10^3 \text{ U/ml}$)	56.4 \pm 12.9	56.3 \pm 7.0	30.7 \pm 3.8	29.7 \pm 8.0	1.44 \pm 0.5	1.52 \pm 0.8	0.96 \pm 0.4	0.75 \pm 0.4

* Values represent concentration in micromolar (cell-free) or nanomoles per 5×10^6 cells presented as mean \pm SD of three to seven independent experiments performed in duplicates or single determinations. † Dialyzed. ND, not detectable.

Table II. α -TOH and CE-OOH Levels, and Relative Electrophoretic Mobility of LDL after Incubation in Ham's F-10 for 24 h in the Absence or Presence of PBMC or Purified Monocytes*

F-10	Control (6)	LDL only		LDL + PBMC			LDL + monocytes	
		No addition (6)	+3HAA [‡] (4)	No addition (5)	rIFN γ (4)	+3HAA [‡] (3)	No addition (2)	+3HAA [‡] (2)
α -TOH (%) [§]	100 ^a	82.1 \pm 17.7 ^b	87.7 \pm 17.8 ^{a,c}	16.6 \pm 23.8 ^d	46.2 \pm 34.6 ^{b,c}	87.6 \pm 8.7 ^{a-c}	41.7	88.4
CE-OOH (μ M)	0.12 \pm 0.21 ^a	12.7 \pm 10.7 ^b	0.05 \pm 0.04 ^{a,b}	28.0 \pm 10.3 ^c	14.6 \pm 15.4 ^{a,b}	0.00 \pm 0.0 ^{a,b}	41.6	0.09
Relative electrophoretic mobility	1.00 ^a	1.24 \pm 0.18 ^b	1.08 \pm 0.17 ^{a-c} (5)	1.47 \pm 0.18 ^c	1.24 \pm 0.20 ^b	1.14 \pm 0.13 ^a	1.44 (1)	1.11 (1)

Results are expressed as means \pm SD (numbers in parentheses represent number of independent experiments). Values within rows sharing the same letter(s) are not significantly different from each other.

* Cells (5×10^6 PBMC/ml or 10^6 monocytes/ml) preincubated in RPMI 1640 + 15% human serum for \approx 2–3 h at 37°C before 24-h exposure to LDL with and without additives. [‡] Final concentration was 10 μ M (includes data from experiments in F-10 medium without added Trp).

[§] Initial concentration (= 100%) was 1.95 ± 0.84 μ M (mean \pm SD) ranging from 0.9 to 2.91 μ M. ^{||} CE-OOH of control LDL was determined after sterile-filtration prior to the incubation. The level of CE-OOH of the freshly prepared LDL before gel-filtration and any further treatment was 0.007 ± 0.01 μ M (mean \pm SD, n = 6), consistent with the very low concentrations found in rapidly isolated LDL (10, 34).

ble II). An even greater effect was observed, when the potent antioxidant 3HAA (17) was added to the cells at the beginning of the incubation and in the absence of IFN γ . In this case, concentrations of CE-OOH and α -TOH as well as LDL's relative electrophoretic mobility were indistinguishable from the corresponding control values. Monocyte-mediated oxidative modification of LDL was prevented similarly by the addition of 3HAA (Table II). These results demonstrate that LDL (per)oxidation in F-10 medium proceeds via a chain reaction that is accelerated by human mononuclear cells, but can be inhibited strongly by either addition of micromolar amounts of 3HAA or treatment of the cells with IFN γ .

Since the concentrations of 3HAA used in Table II exceeded those formed by PBMC activated by IFN γ (cf. Table I) we examined the concentration-dependent inhibition of LDL oxidation by 3HAA. As can be seen in Fig. 1, 3HAA inhibited LDL oxidation at very low concentrations, in the presence or absence of cells. In both cases, accumulation of CE-OOH was strongly inhibited by 3HAA concentrations as low as 0.5 μ M, where only \approx 10–20% of the extracellular α -TOH was lost. Addition of Kyn (50 μ M) together with AA (2 μ M) to the cell-free or PBMC-containing system at concentrations similar to those produced by IFN γ -treated cells after 24 h (cf. Table I) did not reduce LDL oxidation significantly (data not shown). Similarly, added ascorbic acid (0.5 μ M) did not significantly inhibit PBMC accelerated LDL oxidation (not shown). These results show that 3HAA at a concentration similar to that produced by IFN γ -treated PBMC strongly inhibits LDL lipid peroxidation.

One approach to assess the extent of participation of oxidative Trp degradation in the observed IFN γ -induced inhibition of PBMC-mediated LDL oxidation in general and that of 3HAA in particular, would be to selectively inhibit 3HAA formation in stimulated mononuclear cells. The latter is dependent on the monooxygenase-catalyzed hydroxylation of Kyn (20) (Scheme I). Since monooxygenases are often flavin-dependent enzymes, we tested whether the flavin analogue DPI could inhibit 3HAA formation. Indeed, DPI inhibited IFN γ -induced formation of 3HAA by PBMC very effectively but showed no significant effect on degradation of Trp and forma-

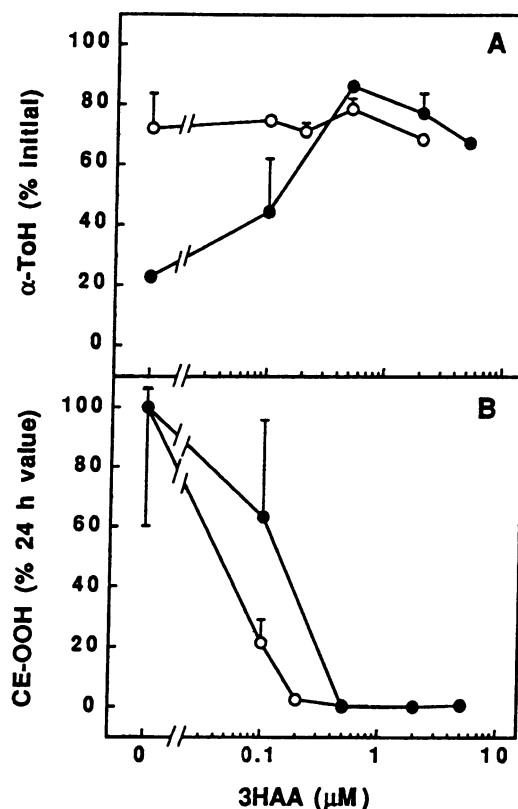


Figure 1. Inhibition of LDL oxidation by 3HAA. LDL (dialyzed) was incubated for 24 h at 37°C in F-10 medium only (○), or in the presence of PBMC (5×10^6 cells/ml, ●). Various amounts of 3HAA were added to the LDL-containing cultures at the beginning of the incubation. LDL oxidation was measured by determination of extracellular α -TOH and CE-OOH as described in Methods. Each value represents the mean \pm SE of triplicate incubations of a typical (of three cell-free and two cell-containing) independent experiment. (A) α -TOH concentration remaining after 24 h of incubation relative to that present prior to incubation (i.e., 0.8 μ M = 100%). (B) CE-OOH concentration, expressed relative to that detected after 24 h in the absence of 3HAA (i.e., 3.5 and 10.7 μ M in the absence and presence of cells respectively).

tion of Kyn and anthranilic acid by these cells at concentrations up to $\approx 1 \mu\text{M}$ (Fig. 2). Comparable results were obtained with MDM and whether LDL was present or not (not shown).

We next tested whether the inclusion of DPI at a concentration that "selectively" inhibited 3HAA formation could reverse IFN γ -mediated inhibition of PBMC-facilitated LDL oxidation. The time-dependent analysis of such examination is shown in Fig. 3. Incubation of LDL in the presence of PBMC resulted in rapid consumption of extracellular α -TOH after an initial lag phase of ≈ 6 h (Fig. 3 A). This was paralleled by a gradual loss from the medium of C18:2, the major polyunsaturated core lipid and therefore substrate for oxidation in LDL (Fig. 3 B). Concomitant with the decrease in α -TOH and C18:2, extracellular CE-OOH increased (Fig. 3 C), accounting for $82.7 \pm 43.6\%$ (mean \pm SD, $n = 4$) of the C18:2 lost. CE-OOH represent those lipid hydroperoxides that are derived from polyunsaturated fatty acids in LDL esterified to cholesterol. However, since the cholesteryllinoleate hydroperoxide detected during oxidation of human LDL usually accounts for more than 85% of the total CE-OOH formed (33) the above calculation (i.e., CE-OOH formed per C18:2 lost) gives a reasonably accurate estimate of the degree of conversion of nonoxidized core lipids into the corresponding hydroperoxides. Importantly, in the absence of IFN γ , addition of DPI did not alter cell-mediated LDL oxidation, as seen from the rates of α -TOH/C18:2 consumption and CE-OOH formation (Fig. 3). Likewise, LDL oxidation in F-10 medium alone was not altered by the addition of DPI (not shown). In contrast, incubation of LDL with PBMC in the presence of IFN γ prevented or almost completely inhibited the consumption of C18:2 and accumulation of CE-OOH, and reduced the loss of α -TOH by $\approx 50\%$ (Fig. 3). In some cases the amounts of CE-OOH detected in the presence of IFN γ were similar to or even less than

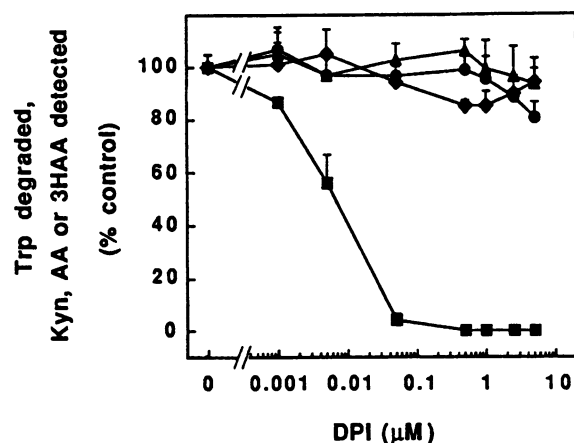


Figure 2. DPI-mediated "selective" inhibition of IFN γ -induced formation of 3HAA by PBMC. Cells (5×10^6 /ml) were incubated in the presence of IFN γ (10^3 U/ml) for 48 h at 37°C in the absence or presence of various concentrations of DPI before supernatants were analyzed for Trp (\bullet), Kyn (\blacktriangle), AA (\blacklozenge) and 3HAA (\blacksquare) as described in Methods. Ethanol at the highest concentration used as solvent for DPI (0.5%) had no significant effect on IFN γ -induced Trp metabolism. Values represent means \pm SD of three to seven independent experiments and are expressed as percentage of the control incubation, i.e., in the absence of DPI. The absolute control values (i.e., 100% values) for Trp and its metabolites are indicated in Table I.

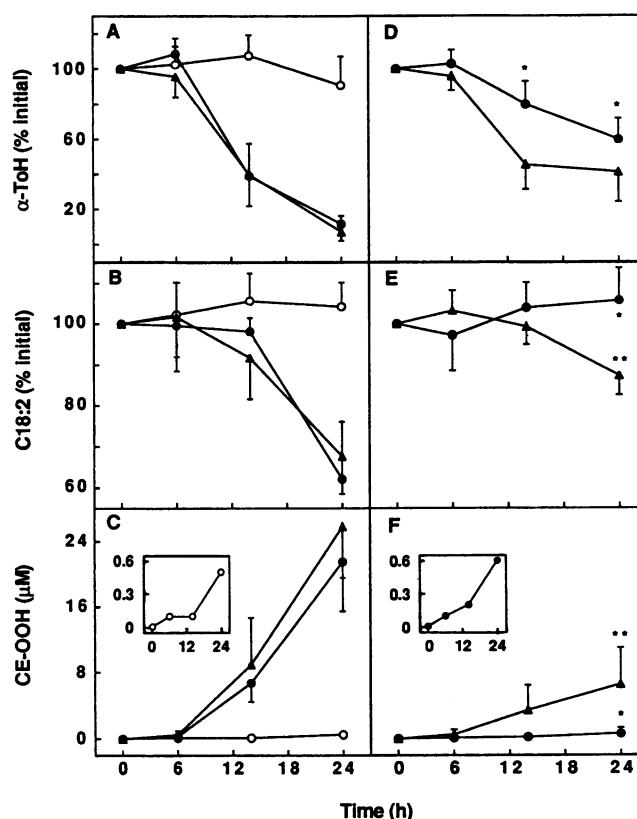


Figure 3. Time-dependent analysis of the inhibition of cell-mediated LDL oxidation by IFN γ and its partial reversal by DPI. LDL (dialyzed) was incubated in the absence (\circ), or presence (solid symbols) of untreated (A–C) or IFN γ -treated (10^3 U/ml, D–F) PBMC (5×10^6 cells/ml, 2 ml final volume) at 37°C in the absence (\bullet) or presence (\blacktriangle) of 20 nM DPI added at the beginning of the incubation. Aliquots of the medium were analyzed for α -TOH (A and D), C18:2 (B and E), and CE-OOH (C and F) using cholesterylbenzoate as the internal standard as described in Methods. Initial concentrations (means \pm SE) for α -TOH, C18:2, and CE-OOH were 1.25 ± 0.3 (ranging from 0.55 to 1.83), 95.6 ± 19.0 (from 54.9 to 140.8), and $0.008 \pm 0.005 \mu\text{M}$, respectively. Each value represents the mean \pm SE of 4 independent experiments performed in duplicates. (Insets) CE-OOH accumulation during cell-free (C) and IFN γ -inhibited cell-mediated (F) LDL oxidation presented using an enlarged scale. *Significantly different to corresponding value of untreated cells (minus IFN γ). **Significantly different to IFN γ -treated cells in the absence of DPI.

those observed in the appropriate cell-free incubation (Fig. 3, E and F insets). Addition of 20 nM DPI significantly reversed part of the inhibitory effect of IFN γ on consumption of C18:2 and accumulation of CE-OOH (Fig. 3, E and F). Since only partial reversal was observed with this very low concentration of DPI, we tested whether higher inhibitor concentrations caused a more pronounced effects. Indeed, addition of 100 nM DPI, a concentration that totally inhibited 3HAA formation (Fig. 2), reversed the ability of IFN γ to inhibit PBMC-mediated LDL oxidation between 51% and 100%, depending on the parameter of LDL oxidation measured (Table III).

As a second approach to assess whether Trp metabolism along the Kyn pathway contributed to the observed inhibitory action of IFN γ on cell-mediated LDL oxidation, we used

MDM in Ham's F-10 medium that was not supplemented with Trp. Commercial F-10 medium contains only low micromolar concentrations of Trp, so that significant amounts of 3HAA can not be formed by cells even when IDO is induced by treatment with IFN γ . Indeed, addition of IFN γ to MDM cultured in nonsupplemented medium did not result in detectable Kyn or 3HAA (data not shown) and had no effect on MDM-mediated LDL oxidation (Fig. 4). In contrast, IFN γ treatment of MDM in Trp-supplemented F-10 medium resulted in formation of micromolar amounts of 3HAA (ranging from 1 to 30 μ M; depending on the cell numbers and IFN γ dosage used) and almost complete inhibition of LDL oxidation (Fig. 4). Similar results were obtained with PBMC (not shown). Addition of Trp (75 μ M) alone to either the MDM or cell-free incubations only marginally affected LDL oxidation, whereas inclusion of DPI in the MDM/Trp/IFN γ system caused substantial inhibition of Trp metabolism and reversion of the cytokine's inhibitory effect on LDL oxidation (not shown). Together, these results strongly support an important role of 3HAA formation in IFN γ -mediated inhibition of LDL oxidation by human monocytes/macrophages.

Discussion

We have examined the potential inhibition by IFN γ of the early stages of human monocyte and macrophage-mediated LDL oxidation in Ham's F-10 medium containing physiological amounts of added Trp, measuring the consumption of the lipoprotein's major antioxidant (i.e., α -TOH) and targets for oxidation (C18:2 and C20:4) together with the accumulation of CE-OOH, the primary and major oxidation product. Our results show that as with murine macrophages, IFN γ strongly attenuates cell-mediated LDL oxidation by human monocytes/macrophages. Unlike the murine system however, IFN γ -mediated inhibition of LDL oxidation by human monocytes/macrophages does not appear to be linked to NO biosynthesis, as this pathway is not induced, at least under the experimental conditions used in this study. In contrast, our results indicate a

Table III. Reversal of the Inhibitory Effect of IFN γ on PBMC-mediated Oxidation of LDL by DPI

	α -TOH*	CE-OOH [†]	CE _{PUFAs} [‡]
	% remaining	μ M	% degraded
PBMC	0	29.5 \pm 2.8	79.5 \pm 2.8
PBMC + IFN γ	20.7 \pm 4.4	5.7 \pm 2.5	10.8 \pm 10.0
PBMC + IFN γ + DPI	0	18.0 \pm 2.5	67.0 \pm 5.0
F-10 alone	5.0 \pm 5.1	13.5 \pm 5.8	39.8 \pm 3.5

DPI at 100 nM inhibited 3HAA formation by 100% consistent with results shown in Fig. 2. 3HAA level in PBMC + IFN γ was 0.95 μ M \pm 0.2. Results are presented as means \pm SD of an individual experiment carried out in triplicate and representative of 2 independent experiments.

* Initial α -TOH concentration (100%) was 1.7 μ M \pm 0.3. [†] CE-OOH level before addition to culture medium was 0.01 μ M. [‡] CE_{PUFAs} refer to C18:2 plus C20:4. ^{||} Cells (5×10^6 PBMC/ml) were incubated in Ham's F-10, supplemented with 75 μ M L-Trp, for 24 hr in the presence of LDL and the presence or absence of IFN γ (1000 U/ml).

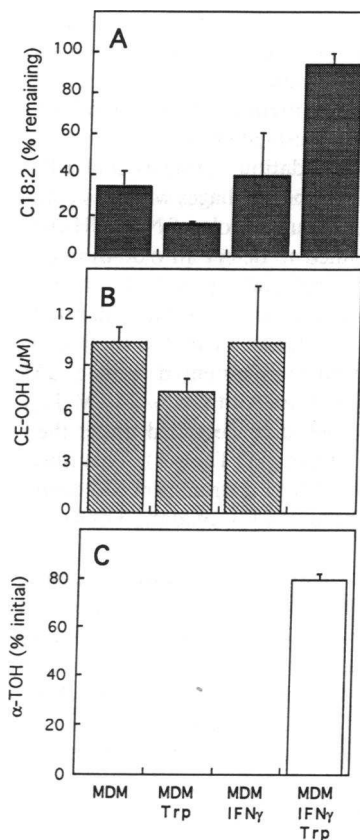


Figure 4. Inhibition of MDM-mediated LDL oxidation by IFN γ is dependent on the presence of Trp in the culture medium. LDL (100 μ g protein/ml) was incubated for 24 h in Ham's F10 medium supplemented (75 μ M) or not supplemented with Trp and in the absence and presence of MDM (3×10^6 cells/well) and IFN γ (1,000 U/ml) before the concentrations of cholesteryllinoleate (A), CE-OOH (B), and α -TOH (C) in the medium were determined (see Methods). Before exposure to LDL, MDM were pre-cultured for 20 h in RPMI 1640 medium containing 10% heat-inactivated human serum in the presence or absence of IFN γ (250 U/ml). The results show the means \pm SD of triplicate determinations from one out of three independent experiments. In all three experiments IFN γ -induced inhibition of MDM-mediated LDL oxidation was

dependent on Trp supplementation of the medium. In the absence of IFN γ addition of Trp to MDM had small but variable effects on cholesteryllinoleate consumption and CE-OOH formation, but did not affect α -TOH consumption. Initial concentrations (100% values) of C18:2 and α -TOH are indicated in the legend to Fig. 3.

protective role for oxidative Trp metabolism and 3HAA formation in this process.

The evidence for the participation of oxidative Trp metabolism and 3HAA formation in IFN γ -mediated inhibition of human monocyte/macrophage-mediated LDL oxidation is based on the following: Firstly, exposure of these cells to IFN γ -induced oxidative Trp metabolism along the Kyn pathway that resulted in the release of low micromolar amounts of 3HAA. Secondly, this aminophenolic metabolite is a powerful antioxidant (17, 46): it strongly inhibited LDL oxidation when added to both cell-free and cell-containing systems in the absence of IFN γ and at concentrations similar to those formed by IFN γ -treated mononuclear cells in vitro. Thirdly, incubation of LDL and IFN γ with PBMC or MDM under conditions that resulted in oxidative Trp degradation with concomitant formation of 3HAA, inhibited LDL oxidation to an extent similar to that observed with exogenously added 3HAA. Fourthly, "selective" inhibition of IFN γ -induced formation of 3HAA by inclusion of the flavoprotein inhibitor DPI (47, 48) reversed the inhibitory action of this cytokine on mononuclear cell-facilitated LDL oxidation. Finally, the inhibitory activity of IFN γ on MDM-mediated LDL oxidation was dependent on the presence of physiological amounts of Trp in the medium that did

allow detectable levels of 3HAA to be formed. Together these results show that 3HAA is a major contributing factor in the IFN γ -mediated inhibition of monocyte/macrophage-mediated LDL oxidation in transition metal containing medium.

Previous work by others has investigated a modulatory role of IFN γ on cell-mediated LDL oxidation. Cathcart et al. (49) treated human monocyte-derived macrophages with this cytokine to examine a possible contributory role of NADPH-oxidase on LDL oxidation, but failed to detect an oxidation-enhancing effect. Hurt-Camejo and colleagues (50) showed (though did not discuss) results consistent with an inhibitory action of IFN γ on LDL lipid oxidation mediated by human MDM in RPMI 1640 medium supplemented with 5 μ M CuSO $_4$ (their Table IV). This medium contains \approx 25 μ M Trp, most of which would be expected to be degraded under their conditions with formation of 3HAA (cf. Table I). This raises the possibility that IDO-initiated Trp degradation too might have contributed to the inhibition of LDL oxidation observed by these authors.

IFN γ is an important modulator of the immune response, affecting many cellular functions of monocytes/macrophages (51). Among these effects are an increased capacity of phagocytes to produce reactive oxygen species upon appropriate stimulation and, conversely, the induction of several metabolic pathways that can be regarded as providing additional antioxidant protection within and surrounding the cells (Scheme I). Here we investigated induction of IDO, the initial and O $_2$ ⁻-requiring enzyme (52) of the Kyn pathway that can give rise to hydroxylated, antioxidant active metabolites (17) (Scheme I). With one exception (i.e., A 498 kidney carcinoma cells), human monocytes/macrophages are the only cells known to produce and release 3HAA upon IFN γ stimulation (20, 21). As mentioned above, evidence for the participation of Trp metabolism and 3HAA formation in particular, in the IFN γ -mediated inhibition of LDL (per)oxidation is based in part on the fact that inclusion of submicromolar amounts of DPI reversed the inhibition in consumption of α -TOH, C20:4 and C18:2, and formation of CE-OOH. The extent of this reversal increased with increasing concentrations of DPI used (cf. Fig. 3 and Table 4).

We have used DPI at low concentrations in an attempt to inhibit formation of 3HAA without affecting other enzymatic reactions that are known to be inhibited by higher concentrations of DPI and may affect LDL oxidation. Thus, the final concentration of DPI used (20–100 nM) is some two orders of magnitude lower than that required for inhibition of NADPH oxidase (53), the superoxide anion radical-producing enzyme suggested to be involved in human PBMC-mediated LDL oxidation (54). Also, inhibition of NO synthase in murine macrophages requires somewhat higher DPI concentrations than used in our experiments (12, 14, 48). In any case, an involvement of this enzyme in the downregulation of LDL oxidation by IFN γ as reported for mouse peritoneal macrophages (12, 13), is unlikely to be important in the human system, as neither accumulation of nitrite nor degradation of arginine from the culture medium were observed. In contrast, our studies using Trp poor medium clearly demonstrate a requirement for Trp metabolism in the inhibitory activity seen by IFN γ that is affected by DPI.

Incubation of LDL in the presence of PBMC resulted in detectable extracellular CE-OOH after an initial lag period of

\approx 6 h, when significant levels of α -TOH were still present (Fig. 3). The observation that α -TOH was consumed during rather than before the onset of lipid peroxidation is consistent with our previous observations on cell-free, radical-mediated LDL oxidation (10, 12, 44) and also transition metal-induced oxidation (45). It is also consistent with a role for α -TOH-mediated peroxidation (45) during PBMC-mediated LDL oxidation (in the absence of suitable reductants for α -tocopheroxyl radical) and LDL lipid peroxidation proceeding in a chain reaction. In fact, preliminary studies showed that enrichment of LDL with α -TOH enhanced MDM-mediated lipid hydroperoxide formation during the early stages of lipoprotein oxidation in F-10 medium (Dr. Thomas, Dr. Jiri Neuzil, and Dr. Stocker, unpublished observations).

A striking finding of our study was the efficacy with which very low concentrations of 3HAA inhibited LDL oxidation. Although it is difficult to directly compare our results with those from other groups, 3HAA is clearly one of the most efficient LDL antioxidants known at present. Hence, an understanding of the mechanism(s) involved in this inhibition will be important. 3HAA likely inhibited LDL oxidation at an early stage of radical-induced chain oxidation of the lipoprotein, whether cells were present or not. The possibility that 3HAA chelates and makes the transition metals in Ham's F-10 medium unavailable to participate in LDL oxidation, can be ruled out for several reasons: Firstly, separate *in vitro* studies (Dr. Christen and Dr. Stocker, unpublished observations) showed that 3HAA (5 μ M) is unable to inhibit F-10 medium-catalyzed (auto)oxidation of ascorbate. Even when present at up to a 50-fold molar excess over the metal, 3HAA does not inhibit Cu $^{2+}$ or Fe $^{3+}$ -catalyzed oxidation of ascorbate in phosphate buffer, pH 7.0. Secondly, neither the absorption nor fluorescence spectra of 3HAA in solution are altered by the addition of either Cu $^{2+}$ or Fe $^{3+}$, suggesting that the aminophenol does not complex with these metals under these conditions. This is consistent with previous studies demonstrating that stable complexes of 3HAA with Cu $^{2+}$ or Fe $^{3+}$ are formed at alkaline pH only, i.e., when both the amino and hydroxyl groups of 3HAA are deprotonated (the pK $_a$ values for amino and hydroxyl groups are 5.2 and 10.1, respectively) (55). Thirdly, the results in Fig. 1 show that LDL oxidation is inhibited by concentrations of 3HAA that are clearly below (i.e., submicromolar) those of the transition metals present in Ham's F-10. (i.e., \approx 3 μ M iron plus 0.01 μ M copper).

Alternatively, 3HAA may protect LDL from oxidation by reducing (or eliminating) the tocopheroxyl radical produced from α -TOH, and/or maintaining (LDL-associated) ubiquinol-10 in the reduced, antioxidant active form. Either or both of these reactions would provide powerful antioxidation (10, 11, 44, 45). A reaction of tocopheroxyl radical with aqueous reductants (antioxidants) has been reported for ascorbate and bilirubin (56, 57). While we are currently investigating these possibilities, it is clear from our results that if 3HAA operates in one or both of the latter modes, components of F-10 medium would have to maintain 3HAA in the reduced state because it is not consumed substantially while providing antioxidation. This opens the intriguing possibility that IFN γ modulates the production and/or release by monocytes/macrophages of a "catalyst" for LDL antioxidation. Low concentrations of such a catalyst would be sufficient to provide substantial additional protection by maintaining LDL's antioxi-

dant(s) in the reduced state through common and readily available reductants which themselves are not efficient antioxidants, and can not interact with LDL's α -TOH.

In addition to the protective antioxidant action of IFN γ , this cytokine is likely to have other effects relevant to atherosclerosis. Thus, treatment of human MDM with IFN γ causes inhibition of synthesis and secretion of lipoprotein lipase (58) as well as expression of the scavenger receptor and the development of foam cells in vitro (59). The suppressive effect of IFN γ on production of platelet-derived growth factor by mononuclear phagocytes (60) may also be important in the control of smooth muscle cell proliferation, which is a major event in the development of the atherosclerotic plaque. It has been demonstrated that IFN γ administration regulates vascular smooth muscle proliferation and inhibits lipase activity in vivo (61). Lymphocytes and monocytes/macrophages are major cell types present in the healthy intima at about the same ratio as in circulation (25). As a fatty streak forms and progresses into a lesion, the relative contribution of monocytes/macrophages increases as does the absolute number of cells (25). IFN γ is likely to be present in atherosclerotic lesions, as evidenced by the presence of MHC class II antigens and other specific activation markers on cells found in those areas (23, 25). Induction of IDO with release of 3HAA thus appears feasible and may either alone, or together with the aforementioned activities, influence the development of atherosclerosis as well as other inflammatory processes.

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