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Dual effects of oxidized low-density lipoprotein on immune-stimulated nitric oxide and prostaglandin release in macrophages

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Abstract

Oxidized low-density lipoprotein (LDL) is currently regarded as a tentative key player in atherosclerosis by virtue of its ability to induce intracellular lipid accumulation and to modulate cell functions in the vessel wall. We previously demonstrated that inducible nitric oxide (NO) synthase activity is attenuated in lipid-laden J774 macrophages obtained by incubation with oxidized LDL 200 μg ml^{-1} for 24 h. In the present study we investigated the effect of oxidized LDL in a lower concentration (20 μg ml^{-1}) or for a shorter time (6 h) and the possible mediator role of prostaglandin E_2 and prostacyclin. Prostaglandins and the NO synthase metabolites citrulline and nitrite were elevated in the 24 h supernatant after immune stimulation with interferon-γ 100 U ml^{-1} with or without lipopolysaccharide 10 μg ml^{-1}. Pretreatment with oxidized LDL 20 μg ml^{-1} for 18 h decreased nitrite release by 31 $\pm$ 2%, whereas prostaglandin production was not affected. A 6 h pre-exposure to 200 μg ml^{-1} had an opposite effect: it significantly potentiated interferon-γ-stimulated prostaglandin E_2 (10-fold), prostacyclin (7-fold), nitrite (1.5-fold), and citrulline (2.4-fold) release. Indomethacin 10 μM abolished the prostaglandin production and largely prevented the oxidized LDL-dependent increase in NO synthase activity. Acetylated LDL was without effect. The data show that the immune-induced release of NO is potentiated or suppressed, depending on the conditions of exposure to oxidized LDL. The potentiation due to short, high-dose exposure is partly mediated by prostaglandins since indomethacin inhibited both processes.

Keywords: Low-density lipoprotein, oxidized; Nitric oxide (NO); Prostaglandin; Macrophage; Atherosclerosis

1. Introduction

The early stage of atherosclerosis is characterized by the infiltration of monocytes into the subendothelial space (Gerrity, 1981), a process in which oxidized low-density lipoprotein (LDL) may be involved (Quinn et al., 1987; Cushing et al., 1990). Oxidation of LDL has been demonstrated to occur in vivo (Palinski et al., 1989), possibly as a result of macrophage 15-lipoxygenase activity (Ylä-Herttuala et al., 1991). The subsequent scavenger receptor-mediated uptake (Kurihara et al., 1991) of oxidatively modified LDL by macrophages results in intracellular cholesterol accumulation, thus contributing to the formation of the characteristic 'foam cells' in atherosclerotic lesions. Furthermore, oxidized LDL is chemotactic (Quinn et al., 1987) and mitogenic (Yui et al., 1993) for monocytes, and may also affect atherogenesis indirectly by modulating several macrophage functions. For instance, oxidized LDL has been reported to induce interleukin-1β (Ku et al., 1992) and to suppress the expression of several lipopolysaccharide-stimulated inflammatory genes such as tumour necrosis factor-α, interleukin-1α (Hamilton et al., 1991), interleukin-1β and interleukin-6 (Fong et al., 1991). We previously demonstrated that immune-stimulated nitric oxide (NO) production is inhibited in lipid-laden J774 macrophages obtained by incubation with oxidized LDL 200 μg ml^{-1} for 24 h (Jorens et al., 1992; Matthys et al., 1994). In macrophages, NO is formed by the inducible NO synthase, which the cells express upon immune stimulation (Marletta et al., 1988; Nathan, 1992). Recently it has been discovered that activated macrophages also express an inducible cyclooxygenase-2 in addition to the constitutive

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enzyme cyclooxygenase-1 responsible for basal prostaglandin production (Lee et al., 1992). Cyclooxygenase-2 is expressed soon after stimulation, reaching maximal activity 16 h later, whereas cyclooxygenase-1 is unaffected. The aim of the present study was 2-fold. First, we wanted to extend the previous findings (Jorens et al., 1992) by investigating whether oxidized LDL in a lower concentration (20 μg ml^{-1} vs. 200 μg ml^{-1}) or after a shorter exposure time (6 h vs. 24 h) still affects NO synthase activity. Second, we also studied the influence of oxidized LDL exposure on prostacyclin and prostaglandin E_2 production since both are reported to suppress inducible NO synthase induction in J774 macrophages (Marotta et al., 1992).

2. Materials and methods

2.1. Materials

All cell culture media and supplements were from Gibco (Paisley, UK). Lipoprotein deficient serum, LDL, and acetylated LDL were prepared as described. Oxidized LDL was obtained by Cu^{2+} oxidation of LDL (Matthys et al., 1994). Briefly, LDL 200 μg ml^{-1} was incubated for 16 h at 37°C with $CuCl_2$ 6.4 μM, then dialyzed after addition of 200 μM EDTA and concentrated to $\pm$ 1.5 mg ml^{-1}. Recombinant rat interferon-γ was purchased from Holland Biotechnology (Leiden, Netherlands), reagents for the protein assay were from Pierce (Rockford, IL, USA), reagents for the cholesterol assay were from Boehringer Mannheim (Mannheim, Germany), lipopolysaccharide (*Salmonella typhosa*), and prostaglandin E_2 and all other reagents were from Sigma (St. Louis, MO, USA). [^{3}H]6-keto-Prostaglandin $F_{1\alpha}$ and [^{3}H]prostaglandin E_2 were obtained from New England Nuclear (Bad Homburg, Germany). Prostacyclin was a gift from the Wellcome Research Laboratories (Beckenham, UK). Indomethacin was given by Merck, Sharp and Dohme (Brussels, Belgium).

2.2. Culture of J774 macrophages and lipoprotein pretreatment protocols

J774 murine macrophage-like cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and antibiotics (penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1}). Cells were seeded (10^6/well) in 12-well plates, washed with warm DMEM after 2 h to remove non-adherent cells, and subjected to two different lipoprotein pretreatment protocols before stimulation 24 h later. Cells were pretreated with 20 μg ml^{-1} oxidized LDL or acetylated LDL for 18 h or with 200 μg ml^{-1} for 6 h in medium containing 10% lipoprotein-deficient serum and 100 U ml^{-1} polymyxin B. Unexposed cells and acetylated LDL-exposed cells served as controls, since both modified lipoproteins are avidly taken up by macrophages. After lipoprotein pretreatment, cellular cholesterol and protein were determined as described (Matthys et al., 1994). In parallel culture plates, the cells were washed and stimulated with interferon-γ 100 U ml^{-1} with or without lipopolysaccharide 10 μg ml^{-1}. After 4 h, samples of the supernatant were taken for determination of 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2. After 24 h, nitrite, citrulline, 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 were measured in the cell-free supernatant. After the remaining cell monolayer was washed with phosphate-buffered saline, cell protein was precipitated with 5% trichloroacetic acid, dissolved in 0.1 N NaOH plus 0.5% sodium dodecylsulphate, and then determined by the bicinchoninic acid assay (Smith et al., 1985). Experiments were also performed in the presence of indomethacin 10 μM, added during lipoprotein pretreatment and stimulation. Additionally, the effect of exogenous prostaglandins on immune-stimulated NO synthase activity was investigated by the addition of prostacyclin and prostaglandin E_2 at different concentrations (0.03–30 nM).

2.3. Determination of NO synthase activity and prostaglandin production

NO synthase activity was assessed by measuring nitrite, a stable NO metabolite, and L-citrulline in the cell-free supernatant (Matthys et al., 1994) 24 h after stimulation. The detection limit of both assays is 1 μM. Prostacyclin was assessed by a specific and sensitive (detection limit 0.2 nM) radioimmunoassay (RIA) for its stable metabolite 6-keto-prostaglandin $F_{1\alpha}$ (Bult et al., 1985). Prostaglandin E_2 was measured by a similar but less sensitive (detection limit 2 nM) RIA. Dose interpolation was done with a four-parameter logistic function (Dudley et al., 1985), using the IBM-PC RIA data reduction package provided by M.L. Jaffe (Silver Spring, MD, USA).

2.4. Statistical analysis

Values are expressed as the means $\pm$ S.E.M. of four different experiments. Differences between the means were determined by one-way analysis of variance (ANOVA) followed by the Bonferroni test. A P value less than 0.05 was considered significant.

3. Results

3.1. Cellular cholesterol and protein after lipoprotein pretreatment

Exposure to acetylated LDL or oxidized LDL did not cause cell loss, as judged from total cell protein measurements (Table 1). Cellular cholesterol was raised significantly upon exposure to lipoprotein under both conditions

Table 1
Effect of oxidized LDL and acetylated LDL pretreatment (20 μg ml^{-1} for 18 h or 200 μg ml^{-1} for 6 h) on total cell protein and cholesterol

Pretreatment	Protein/well	Cholesterol/ mg protein
No lipoprotein, 18 h	222 ± 11	37 ± 1
Oxidized LDL 20 μg ml^{-1}, 18 h	214 ± 15	53 ± 1 [a,b]
Acetylated LDL 20 μg ml^{-1}, 18 h	237 ± 13	66 ± 3 [a]
No lipoprotein, 6 h	252 ± 13	36 ± 1
Oxidized LDL 200 μg ml^{-1}, 6 h	248 ± 9	60 ± 1 [a,b]
Acetylated LDL 200 μg ml^{-1}, 6 h	253 ± 10	98 ± 3 [a]

Mean of four separate experiments ± S.E.M. [a] Different from non-exposed control. [b] Different from acetylated LDL.

tested, with acetylated LDL being more effectively taken up than oxidized LDL.

3.2. Nitrite and citrulline release

Nitrite or citrulline was not detected in the 24 h supernatant of unstimulated cells (Fig. 1). Stimulation with interferon-γ 100 U ml^{-1} induced inducible NO synthase activity, as indicated by the accumulation of nitrite and citrulline in the medium. Co-stimulation with lipopolysaccharide 10 μg ml^{-1} further increased the production of both NO synthase metabolites. Unstimulated macrophages pre-exposed to lipoproteins did not release nitrite or citrulline. Cells pretreated with a low concentration of modified lipoproteins (20 μg ml^{-1}) for 18 h, and subsequently stimulated with interferon-γ, showed a mild decrease in inducible NO synthase activity (Fig. 1A). This was independent of the type of lipoprotein used. When the stronger combination of interferon-γ + lipopolysaccharide was used,

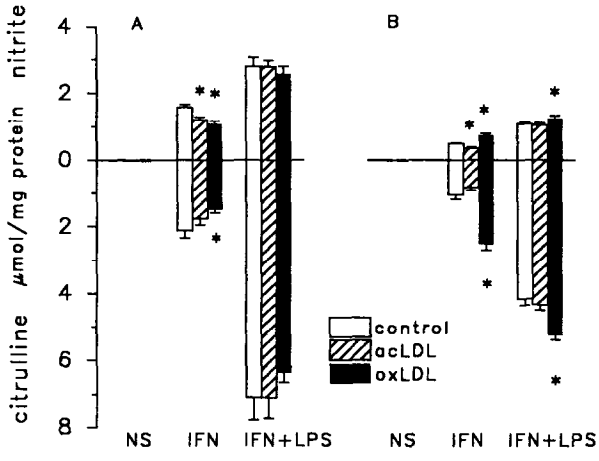

Fig. 1. NO synthase activity in macrophages pretreated with acetylated LDL or oxidized LDL 20 μg ml^{-1} for 18 h (A) or 200 μg ml^{-1} for 6 h (B). Supernatants were assayed for nitrite and citrulline 24 h after stimulation with interferon-γ 100 U ml^{-1} with or without lipopolysaccharide 10 μg ml^{-1}. NS = not stimulated, IFN = interferon-γ, LPS = lipopolysaccharide, acLDL = acetylated LDL, oxLDL = oxidized LDL. * Different from control.

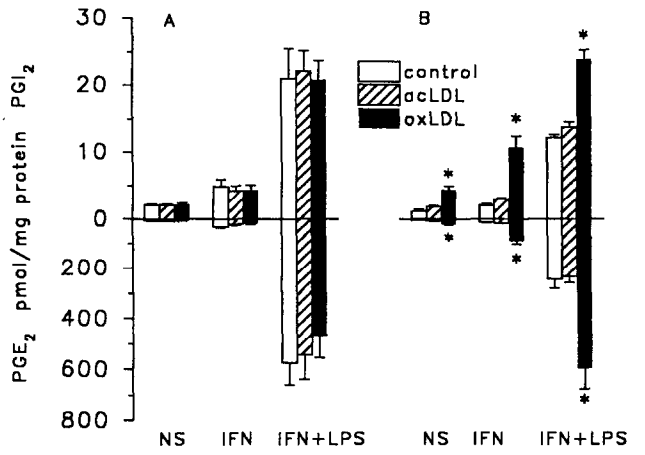

Fig. 2. Prostaglandin production in macrophages pretreated with acetylated LDL or oxidized LDL 20 μg ml^{-1} for 18 h (A) or 200 μg ml^{-1} for 6 h (B). Supernatants were assayed for prostacyclin and prostaglandin E_2 24 h after stimulation with interferon-γ 100 U ml^{-1} with or without lipopolysaccharide 10 μg ml^{-1}. NS = not stimulated, IFN = interferon-γ, LPS = lipopolysaccharide, acLDL = acetylated LDL, oxLDL = oxidized LDL, PGE_2 = prostaglandin E_2, PGI_2 = prostacyclin. * Different from control.

no suppression of inducible NO synthase activity was observed. If the cells were pretreated with a high concentration (200 μg ml^{-1}) of modified lipoproteins for a short time (6 h), and then stimulated with interferon-γ, the modulatory effect on inducible NO synthase activity depended on the type of lipoprotein used, showing opposite effects for acetylated LDL and oxidized LDL (Fig. 1B). Acetylated LDL pretreatment again slightly diminished the release of nitrite and citrulline, whereas pre-exposure to oxidized LDL significantly enhanced it. With the stronger stimulus interferon-γ + lipopolysaccharide, the potentiating effect of oxidized LDL was still observed, although it was less pronounced.

3.3. Prostaglandin production

Prostacyclin and prostaglandin E_2 accumulation in the 24 h supernatant of unexposed, unstimulated macrophages was about the detection limit (Fig. 2). Stimulation of the cells with interferon-γ without or with lipopolysaccharide resulted in a small and strong increase, respectively, in prostaglandin release. Pretreatment of the macrophages for 18 h with low concentrations (20 μg ml^{-1}) of either acetylated LDL or oxidized LDL did not influence basal or immune-stimulated prostaglandin production (Fig. 2A). In contrast, pre-exposure of the cells for 6 h to a 10-fold higher concentration of oxidized LDL, but not acetylated LDL, resulted in significantly higher basal and immune-stimulated prostacyclin and prostaglandin E_2 levels after 24 h (Fig. 2B). The potentiating effect of oxidized LDL pretreatment on immune-stimulated prostacyclin production was already observed after 4 h, with the major increase occurring between 4 and 24 h, and remained highly

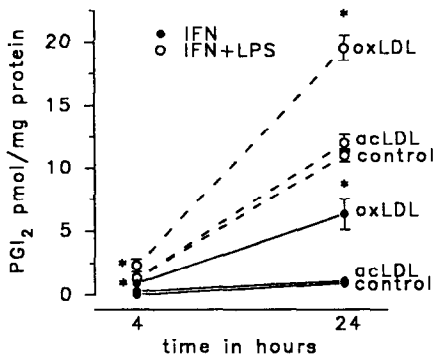

Fig. 3. Immune-stimulated prostacyclin production in macrophages pretreated with oxidized LDL or acetylated LDL 200 μg ml^{-1} for 6 h. Supernatants were assayed 4 and 24 h after stimulation with interferon-γ 100 U ml^{-1} with or without lipopolysaccharide 10 μg ml^{-1}. Basal production is subtracted. IFN = interferon-γ, LPS = lipopolysaccharide, acLDL = acetylated LDL, oxLDL = oxidized LDL, PGI_2 = prostacyclin. * Different from control.

significant also after correction for the enhanced basal production (Fig. 3). Prostaglandin E_2 could not be measured after 4 h because of the lower sensitivity of this assay.

3.4. Effect of indomethacin

Addition of indomethacin 10 μM resulted in complete inhibition of the basal and interferon-γ-stimulated prostaglandin E_2 production in control and oxidized LDL-pretreated cells (Table 2). Prostacyclin production reacted similarly (data not shown). Indomethacin did not significantly affect nitrite and citrulline release in control cells. However, it modulated the up-regulated NO synthase activity due to oxidized LDL pretreatment: the nitrite component which could be ascribed to oxidized LDL was completely prevented and the additional citrulline release significantly decreased. Similar results were obtained when the stronger stimulus interferon-γ + lipopolysaccharide was used.

3.5. Effect of exogenous prostaglandins on NO synthase activity

When prostacyclin or prostaglandin E_2, in concentrations ranging from 0.03 to 30 nM were added together with interferon-$\gamma \pm$ lipopolysaccharide, no effect on nitrite or citrulline production could be detected ($n = 4$, data not shown).

4. Discussion

Many immune modulatory actions, stimulatory as well as inhibitory, have been ascribed to oxidized LDL and may influence atherosclerosis. Also, oxidized LDL is chemotactic (Quinn et al., 1987) and mitogenic (Yui et al., 1993) for monocytes, stimulates monocyte differentiation (Frostegard et al., 1990), prevents egress of tissue macrophages (Quinn et al., 1987) and is taken up by macrophages through scavenger receptors (Kurihara et al., 1991). The close association of oxidized LDL and macrophages in atherosclerotic lesions prompted us to study the effect of oxidized LDL on two macrophage secretory functions, i.e. the release of NO and prostaglandins.

Nitric oxide is formed by the inducible NO synthase, which catalyses the conversion of L-arginine to NO and L-citrulline. NO decomposes to its stable products nitrite and nitrate (Marletta et al., 1988; Nathan, 1992). We assessed inducible NO synthase activity indirectly by measuring the accumulated nitrite and citrulline in the supernatant. Most authors only measure nitrite, based on the assumption that NO is oxidized to nitrite and nitrate in a constant ratio of about 3 : 2 (Marletta et al., 1988). However, NO may escape as gas or bind to macromolecules, and nitrogen oxides other than nitrite/nitrate may be formed (Nathan, 1992) and their ratio may be influenced by the amount of NO produced (Marletta et al., 1988) or the production of reactive oxygen intermediates (Ignarro et al., 1993). Moreover the lipophilic NO may partition in cellular lipid deposits. Therefore citrulline is probably a

Table 2
Effect of 10 μM indomethacin on basal and interferon-γ (100 U/ ml, 24 h) induced prostaglandin E_2 production and inducible NO synthase activity in control and oxidized LDL-pretreated (200 μg/ ml, 6 h) macrophages

Stimulus	Indo [a]	Prostaglandin E_2 pmol/mg protein		Nitrite nmol/mg protein		Citrulline nmol/mg protein	
		Control	oxLDL [b]	Control	oxLDL	Control	oxLDL
NS [c]	–	7 ± 0.2	23 ± 5 [f]	< 3	< 3	< 3	< 3
	+	< 6	< 6	< 3	< 3	< 3	< 3
IFN-γ [d]	–	14 ± 3	91 ± 12 [f]	98 ± 5	148 ± 13 [f]	104 ± 13	253 ± 18 [f]
	+	< 6	< 6	81 ± 4	103 ± 12 [g]	99 ± 16	184 ± 23 [f,g]
IFN-γ + LPS [e]	–	234 ± 36	597 ± 77 [f]	215 ± 14	250 ± 15 [f]	415 ± 20	562 ± 14 [f]
	+	< 6	< 6	174 ± 8	182 ± 11 [g]	374 ± 26	416 ± 24 [g]

Means of four separate experiments ± S.E.M. [a] Indomethacin. [b] Oxidized LDL. [c] Not stimulated. [d] Interferon-γ. [e] Lipopolysaccharide. [f] Different from non-exposed control. [g] Different from oxidized LDL without indomethacin.

better parameter of inducible NO synthase activity than nitrite, especially since the conversion of L-arginine by NO synthase is the only source of citrulline in stimulated macrophages (Benninghoff et al., 1990). Pretreatment of J774 cells with modified lipoproteins clearly influenced the release of nitrite and citrulline. Pre-exposure to low concentrations of acetylated LDL or oxidized LDL for 18 h or a high dose of acetylated LDL for 6 h resulted in a small decrease in nitrite accumulation. These effects were not due to non-specific cytotoxicity of oxidized LDL (Reid et al., 1993), since cell protein was not diminished. The total cell protein correlated very well with the uptake of neutral red by J774 cells, an index of cell viability (data not shown). Only for oxidized LDL was the 30% decrease in nitrite parallelled by a significant 30% decrease in citrulline, suggesting that at least in this case inducible NO synthase activity is truly diminished. It remains to be investigated whether this is due to direct inhibition of enzyme activity by oxidized lipids (Yang et al., 1994) present in oxidized LDL, to inhibition of the induction of NO synthase (Bolton et al., 1994) or to diminished availability of substrate or co-factors. This controversy about the mechanism of the effect of oxidized LDL should be borne in mind when measuring nitrite and citrulline as indirect parameters of NO synthase activity. The diminished release of NO synthase metabolites by prolonged exposure to oxidized LDL is in agreement with our original report (Jorens et al., 1992) and is confirmed by Bolton et al., 1994). In our previous study, the cells were exposed to a high concentration of oxidized LDL for a long time, resulting in more than 50% inhibition of nitrite and citrulline release. Thus, it seems that long-term exposure to oxidized LDL dose dependently inhibits NO synthase formation. This is not caused by lipid accumulation as such since the latter is more important in cells exposed to acetylated LDL in which there is no clear effect on inducible NO synthase activity. Also, exposure to a high dose of oxidized LDL for a short time results in relatively more cholesterol accumulation without a depression of inducible NO synthase. In this case, oxidized LDL even potentiates the inducible NO synthase activity. Thus it seems that macrophages become primed for higher NO production after their first contact with oxidized LDL whereas long-term exposure eventually inhibits inducible NO synthase activity. The initial reaction may constitute a protective mechanism since macrophages, activated to generate NO, have a reduced ability to oxidize LDL (Yates et al., 1992) and NO inhibits the toxicity of oxidized LDL to endothelial cells (Struck et al., 1995). Additionally, the later developing inhibition of NO release may also be protective in view of the cytotoxic effect of NO itself (Walker et al., 1995).

We also measured prostacyclin and prostaglandin E_2 release from unstimulated and immune-stimulated macrophages. The basal output, probably due to cyclooxygenase-1 activity, was low. Stimulation with interferon-γ alone or together with lipopolysaccharide resulted in a small and strong increase, respectively, in prostaglandin accumulation in 24 h. Since immune stimulation induces cyclooxygenase-2 activity without affecting cyclooxygenase-1 (Lee et al., 1992), and since most of the prostaglandin formation occurred between 4 and 24 h after stimulation, the largest part of the accumulated prostaglandins is probably derived from cyclooxygenase-2 activity. It has been reported that pre-exposure of macrophages to acetylated LDL (Mathur et al., 1989) or oxidized LDL (Arai et al., 1992) decreases the immediate, probably cyclooxygenase-1-mediated, prostaglandin production in response to agents like zymosan. We looked at the effects of lipoproteins on immune-stimulated, as discussed above probably cyclooxygenase-2-mediated, prostaglandin release. In our experiments, pre-exposure to low concentrations of oxidized LDL or acetylated LDL did not influence basal (cyclooxygenase-1) or immune-stimulated (cyclooxygenase-2) prostanoid release. However both activities were significantly increased after the relatively short pre-exposure to the higher concentration of oxidized LDL. This is not due to increased delivery of the cyclooxygenase substrate, arachidonate, to the cells because acetylated LDL did not potentiate prostaglandin production even though it was taken up more effectively. The higher prostaglandin production in immune-stimulated, oxidized LDL-pretreated cells was already observed 4 h after stimulation, with the major increase occurring after that time. This suggests that short exposure to a high concentration of oxidized LDL primes the cells for enhanced cyclooxygenase-2 activity.

The concomitant increase in nitric oxide and prostaglandin production after a short exposure to oxidized LDL suggested a possible link between these two macrophage mediators. Although prostaglandin E_2 is usually considered a suppressor of various leucocyte functions, it is also reported to enhance the release of tumour necrosis factor-α from macrophages (Renz et al., 1988), which in turn may be involved in the induction of NO synthase (Jorens et al., 1995). Concerning the influence of prostaglandins on inducible NO synthase activity, all possible effects ranging from stimulation (Gaillard et al., 1991) to inhibition (Marotta et al., 1992; Bulut et al., 1993; Raddassi et al., 1993) to no effect (Imai et al., 1993) have been reported. Moreover, prostaglandin E_2 and prostacyclin may have opposite effects (Tetsuka et al., 1991). Thus the regulation of inducible NO synthase by prostanoids seems very complex. In our experiments, indomethacin tended to decrease NO synthase activity in control cells and acetylated LDL-pretreated cells slightly, but exogenous prostacyclin or prostaglandin E_2 had no effect. This suggests that the influence of prostaglandins on the induction or activity of inducible NO synthase in J774 macrophages under our experimental conditions is rather limited. However, the potentiation of the accumulation of citrulline and nitrite caused by a brief exposure of the cells to oxidized LDL

was significantly decreased and abolished, respectively, by indomethacin, suggesting a role for prostaglandins in the oxidized LDL-dependent up-regulation of inducible NO synthase activity.

In conclusion, in addition to the suppression of inducible NO synthase activity observed after long term exposure of J774 cells to oxidized LDL (Jorens et al., 1992; Bolton et al., 1994), the present experiments also demonstrate that a short pre-exposure of macrophages to oxidized LDL potentiates both prostaglandin production and inducible NO synthase activity. The inhibitory effect of indomethacin on both processes suggests the involvement of prostaglandins in the up-regulation of inducible NO synthase.

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