

ACTIVATED cholesterol-laden macrophages in atherosclerotic lesions are believed to influence the progression of this disease. The induction of nitric oxide synthase (iNOS) activity was investigated in control and cholesterol-laden J774 macrophages, obtained by pre-incubation with oxidized or acetylated low density lipoproteins (oxLDL, acLDL). Loading with oxLDL caused a small induction of NOS activity in unstimulated cells, as indicated by nitrite and citrulline accumulation in the supernatant. However, it suppressed the iNOS activity resulting from stimulation of the cells with lipopolysaccharide with or without interferon- $\gamma$ . AcLDL had no inhibitory effect, indicating that cholesterol accumulation as such was not responsible. Since the induction of NOS in macrophages is inhibited by glucocorticoids, the possibility that a glucocorticoid-like factor, formed during oxidation of LDL, may cause the inhibition, was investigated. However, addition of the glucocorticoid receptor antagonist mifepristone did not prevent the oxLDL-dependent NOS inhibition, indicating that the glucocorticoid receptor is not involved in the suppressive effect of oxLDL.

**Key words:** Foam cell, Lipoprotein, Macrophage, Mifepristone, Nitric oxide synthase

## Oxidized lipoproteins suppress nitric oxide synthase in macrophages: study of glucocorticoid receptor involvement

K. E. Matthys,<sup>1,CA</sup> P. G. Jorens,<sup>1</sup>  
B. Marescau,<sup>2</sup> M. Rosseneu,<sup>3</sup> H. Bult<sup>1</sup>  
and A. G. Herman<sup>1</sup>

<sup>1</sup>Division of Pharmacology and <sup>2</sup>Neurochemistry, University of Antwerp (UIA), Wilrijk, Belgium, and <sup>3</sup>Department of Clinical Chemistry, A.Z. St.-Jan, Brugge, Belgium

CA Corresponding Author

## Introduction

The early stage of atherosclerosis is characterized by the infiltration of monocytes in the subendothelial space.<sup>1</sup> By the unregulated uptake of oxidized low density lipoproteins (oxLDL) through the scavenger receptor,<sup>2</sup> these mononuclear cells then accumulate large amounts of lipids and acquire a so-called 'foamy' appearance.

Macrophages in general produce numerous mediators which play a role in host defence and may therefore also affect the progression of atherosclerotic lesions. A recently identified secretion product of activated rodent macrophages is nitric oxide,<sup>3</sup> which contributes to host defence by its cytolytic and antimicrobial properties. It is synthesized from L-arginine by an inducible form of nitric oxide synthase (iNOS), with L-citrulline as a by-product. The expression of iNOS has been demonstrated in atherosclerotic vessels.<sup>4</sup>

The effect of foam cell transformation of J774 murine macrophages on the induction of iNOS activity has been reported.<sup>5</sup> It could be confirmed that this function is indeed inhibited in cells loaded with oxLDL but not with acetylated low density lipoproteins (acLDL). As this indicates that not lipid accumulation as such, but some constituent of oxLDL might exert an inhibitory action on the induction of iNOS activity, this study was designed to elucidate the underlying mechanism.

Since the induction of iNOS in macrophages is also inhibited by glucocorticoids (GC)<sup>6</sup> and glucocorticoids are synthesized from cholesterol via oxidative pathways, it was hypothesised that GC-like products might be formed during the oxidation of the cholesterol-rich LDL particle. To investigate whether a GC-like factor in oxLDL might be responsible for the observed inhibition of iNOS activity, the glucocorticoid receptor (GCR) antagonist mifepristone was added during lipid loading and stimulation, thus attempting to reverse the observed inhibition.

## Materials and Methods

**Materials:** All cell culture media and supplements were from Gibco Ltd, Paisley, UK. Reagents for the cholesterol assay were from Boehringer Mannheim (Mannheim, Germany), reagents for the protein assay from Pierce (Rockford, IL, USA), sodium nitrite from Merck (Darmstadt, Germany), tetramethoxypropane from Aldrich Chemie (Brussels, Belgium), and Aquacide I from Calbiochem (La Jolla, CA, USA). Interferon- $\gamma$  (recombinant rat IFN- $\gamma$ ) was purchased from Holland Biotechnology (Leiden, The Netherlands). Mifepristone (RU 38 486) was a gift from Roussel UCLAF (Romainville, France). Lipoprotein deficient serum (LPDS), low density lipoprotein (LDL) and acetylated LDL (acLDL) were prepared as

described previously.<sup>7</sup> All other reagents, lipopolysaccharide (LPS, *Salmonella typhosa*) and dexamethasone, were from Sigma Chemical Company (St. Louis, MO, USA).

**LDL oxidation:** LDL 4 mg/ml was dialysed for 24 h against phosphate buffered saline (PBS) to remove EDTA. Oxidation of LDL 200 µg/ml was performed in PBS by addition of CuCl<sub>2</sub> (6.4 µM) during 16 h at 37°C and stopped by adding EDTA (200 µM) and keeping on ice for 1 h. Before and after oxidation, a sample was taken for determination of thiobarbituric acid reactive substances (TBARS) as a measure of lipid peroxidation. Then the oxLDL solution was dialysed for 24 h against PBS plus EDTA to remove the Cu<sup>2+</sup> ions and concentrated to ±1.5 mg/ml by keeping the dialysis bag in Aquacide I powder for ± 8 h.

**Lipid peroxidation assay:** A sample volume of 0.5 ml was mixed with 2 ml 0.25 M HCl containing 3.75% thiobarbituric acid and 15% TCA, and boiled for 15 min. After centrifugation at 1500 g for 15 min, absorbance was read at 532 nm. Results, expressed as nmol malondialdehyde (MDA) equivalents/mg protein, are obtained from a standard curve with tetramethoxypropane, which decomposes to MDA under the assay conditions.

**Culture and lipid loading of J774 cells:** J774 murine macrophage-like cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml). Cells (2 × 10<sup>6</sup>/35 mm dish) were seeded in 6-well plates to obtain a confluent monolayer 24 h later. Subsequently, lipid loading was performed by incubation with oxLDL (200 µg/ml) or acLDL (100 µg/ml) for 24 h in DMEM supplemented with 10% lipoprotein deficient serum and antibiotics. The amount of total cell protein after loading with acLDL or oxLDL was 724 ± 84 µg and 777 ± 148 µg respectively, which is not different from control cells (690 ± 37 µg), indicating that lipid loading did not result in cell loss (*n* = 4). In some experiments polymyxin B (10 µg/ml) was added during loading.

**Determination of total cellular cholesterol and protein:** After lipid loading the monolayers were washed three times with cold PBS to remove serum proteins and lipoproteins. Cholesterol extraction was performed in the plate by adding 5 ml hexane:propan-2-ol (3:2) for 1 h, followed by a brief wash with 1 ml. The solvent was evaporated under reduced pressure and cholesterol and cholesterol esters determined by an enzymatic assay.<sup>8</sup> Total cell protein was determined by the bicinchoninic acid (BCA) method.<sup>9</sup> The wells were exposed to 3 ml trichloroacetic acid (TCA) 5% for 2 h, followed by dissolution of the precipitated proteins in 2 ml 0.1 N NaOH plus 0.5% sodium

dodecyl sulphate. Results are expressed as µg cholesterol/mg protein and percent esters of total cholesterol.

**Stimulation of J774 cells:** Control and lipid-laden monolayers were washed three times with warm DMEM and fresh medium (DMEM without phenol red, 400 µM L-arginine, 10% FCS, antibiotics) was added. Cells were then stimulated with LPS 10 µg/ml with or without IFN-γ 100 U/ml for 48 h.

**Determination of nitric oxide synthase activity:** NOS activity was assessed by measuring nitrite, a stable NO metabolite, and L-citrulline in the cell-free supernatant 48 h after stimulation. The nitrite assay is based on the diazotization of sulfanilic acid by NO, derived from NO<sub>2</sub><sup>-</sup> at acidic pH. Subsequently, coupling with *N*-(1-naphthyl)-ethylenediamine yields a coloured product that is measured spectrophotometrically at 540 nm.<sup>10</sup> L-Citrulline was measured with a Biotronik LC 6000 E amino acid analyser (Biotronok, Maintal, Germany). Results are expressed as nmoles nitrite and citrulline/mg protein. The detection limit of both assays is 1 µM.

**Effect of dexamethasone and mifepristone on NOS activity:** Dexamethasone 10<sup>-5</sup>, 10<sup>-6</sup> and 10<sup>-7</sup> M was added to the control cells together with the stimulus. The ability of mifepristone, a glucocorticoid receptor (GCR) antagonist, to prevent the effects of dexamethasone, was investigated by co-addition (10<sup>-5</sup> and 10<sup>-7</sup> M). Mifepristone 10<sup>-5</sup> and 10<sup>-7</sup> was also added during lipid loading and stimulation of foam cells to assess its ability to prevent the inhibitory effect of oxLDL.

**Statistical analysis:** Values are expressed as the mean ± S.E.M. of four to five experiments. Differences between the means were determined by the Student's paired *t*-test. A *p*-value less than 0.05 was considered significant.

## Results

**LDL oxidation:** LDL contained very low amounts of MDA equivalents before oxidation, indicating that minimal spontaneous oxidation had occurred. Oxidation with CuCl<sub>2</sub> increased the MDA equivalents 42-fold, while final dialysing and concentrating resulted in substantial loss of TBARS (Table 1).

**Table 1:** LDL oxidation, measured as thiobarbituric acid reactive substances (TBARS)

LDL	TBARS (nmol MDA equivalents/mg protein)
Before oxidation	1.5 ± 0.8 ( <i>n</i> = 8)
After oxidation	64.0 ± 4.0 ( <i>n</i> = 8)
After dialysis	6.9 ± 0.7 ( <i>n</i> = 6)

**Foam cell formation:** The total cholesterol content of control macrophages was  $30 \pm 2 \mu\text{g}/\text{mg}$  protein,  $94 \pm 2\%$  was present as free cholesterol. Incubation of J774 cells with acLDL  $100 \mu\text{g}/\text{ml}$  or oxLDL  $200 \mu\text{g}/\text{ml}$  resulted in foam cells with a comparable high amount of total cholesterol,  $101 \pm 10$  and  $92 \pm 17 \mu\text{g}$  cholesterol/mg protein respectively. The percentage of cholesterol esters was 53% and 36% respectively.

**Nitrite and citrulline release by unloaded J774 cells:** Supernatant of unstimulated cells did not contain measurable amounts of nitrite or citrulline (Fig. 1). LPS  $10 \mu\text{g}/\text{ml}$  induced NOS activity, as indicated by the accumulation of  $59 \pm 15$  nmoles nitrite and  $98 \pm 15$  nmoles citrulline/mg protein in the 48 h supernatant. Addition of IFN- $\gamma$  resulted in significantly higher production of these two L-arginine metabolites, especially citrulline. Interferon- $\gamma$  alone was only a very weak stimulus for NOS induction (data not shown).

**Nitrite and citrulline release by J774 foam cells:** Unstimulated acLDL-laden foam cells did not produce nitrite or citrulline (Fig. 1). However, in oxLDL-laden cells NOS activity was induced, resulting in small amounts of nitrite ( $13 \pm 4$  nmol/mg protein) and citrulline ( $12 \pm 5$  nmol/mg protein) in the 48 h supernatant of unstimulated cells.

LPS  $10 \mu\text{g}/\text{ml}$  induced NOS activity in acLDL-laden cells to the same extent as in control cells. In oxLDL-laden cells however, NOS activity was significantly suppressed, with only 38% of nitrite production and 25% of citrulline production compared to control cells. This was also observed for the stronger stimulation with both LPS and IFN- $\gamma$ , although the inhibi-

tion was less pronounced (75% of nitrite release and 33% of citrulline release compared to control cells).

Addition of polymyxin B together with oxLDL during lipid loading did not eliminate the small induction of NOS activity nor the suppression of LPS induced activity in these cells (data not shown).

**Effect of dexamethasone and mifepristone on NOS activity in J774 cells:** In control cells, addition of dexamethasone together with LPS  $10 \mu\text{g}/\text{ml}$  resulted in dose dependent inhibition of nitrite release (Table 2). The inhibitory effect of dexamethasone was reversed in a concentration dependent manner by co-addition of the glucocorticoid receptor antagonist mifepristone (Table 2, Fig. 2). Mifepristone did not affect nitrite production when added alone (data not shown).

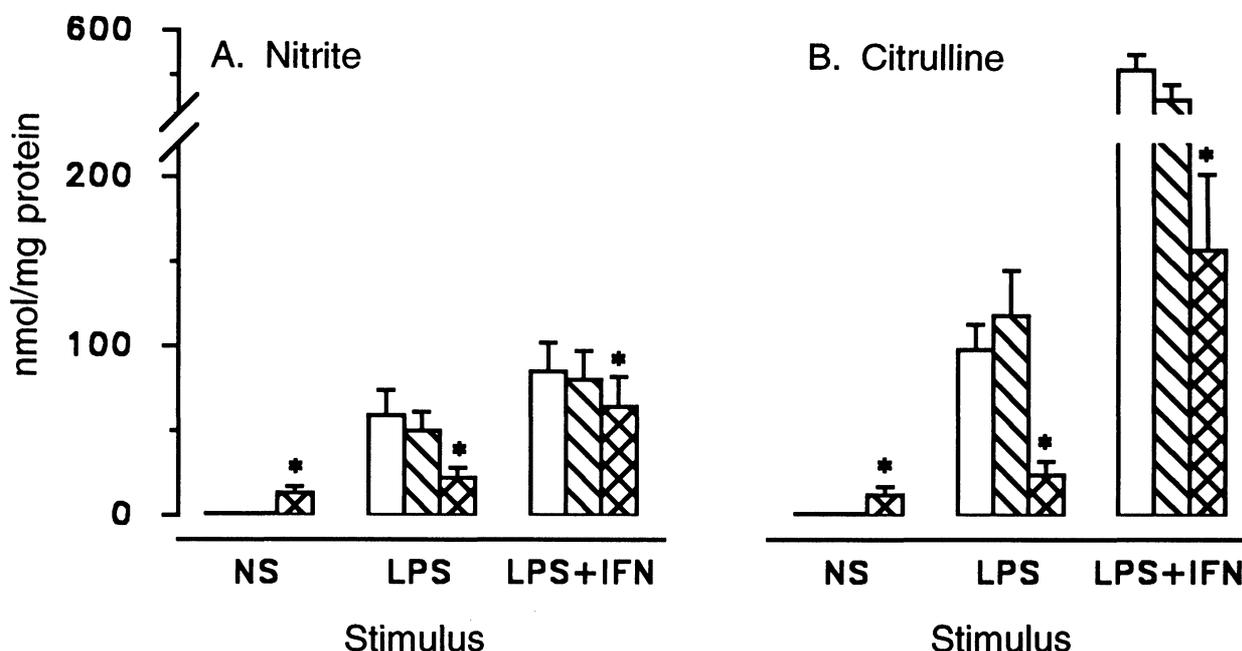
Mifepristone ( $10^{-7}$  M and  $10^{-5}$  M), added during lipid loading and stimulation of the cells, failed to antagonize the inhibition of NOS activity in the oxLDL-laden foam cells (Fig. 2).

**Table 2:** Effect of dexamethasone and mifepristone on LPS induced nitrite release

Concentration of dexamethasone (M)	Control	Concentration of mifepristone	
		$10^{-7}$ M	$10^{-5}$ M
$10^{-7}$	$66 \pm 3$	$77 \pm 2^*$	$89 \pm 4^*$
$10^{-6}$	$34 \pm 3$	$74 \pm 3^*$	$89 \pm 4^*$
$10^{-5}$	$20 \pm 2$	$52 \pm 3^*$	$77 \pm 2^*$

Results are expressed as percent of maximal LPS induced nitrite production  $\pm$  S.E.M. ( $n = 4$ ).

\* $p < 0.05$ , different from dexamethasone alone.



**FIG. 1.** Nitrite (A) and citrulline (B) release by J774 control and foam cells. Nitrite and citrulline were measured in the supernatant of control cells (□), acLDL-laden cells (▨) and oxLDL-laden cells (▩) after 48 h with or without LPS  $\pm$  IFN- $\gamma$ . NS = not stimulated. \* $p < 0.05$  vs. control.

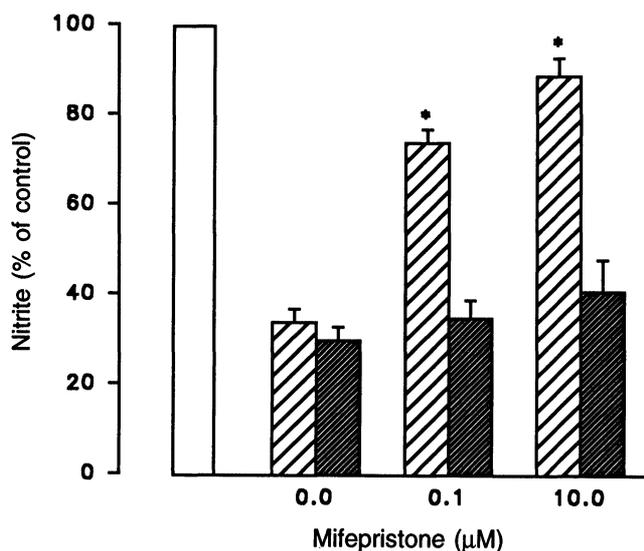


FIG. 2. Mifepristone reverses induced NOS inhibition by dexamethasone ( $10^{-6}$  M) (■) without influencing the suppression by oxLDL (■). Control cells (□) were stimulated with LPS ( $10 \mu\text{g/ml}$ ) and produced  $59 \pm 15$  nmol nitrite/mg protein. Results are expressed as percent of control. \* $p < 0.05$  vs. no mifepristone added.

## Discussion

J774 cells have been used for the study of cholesterol metabolism and lipoprotein handling<sup>7,11</sup> in macrophages. They express the scavenger receptor(s) and thus can take up modified lipoproteins such as acLDL or oxLDL, eventually leading to intracellular cholesterol ester accumulation, the hallmark of foam cells. Several authors have reported the presence of oxLDL in atherosclerotic lesions,<sup>12,13</sup> whereas acLDL is probably not relevant *in vivo*. *In vitro*, both forms of modified lipoproteins induce lipid accumulation in J774 cells, and thus provide an easy-to-use foam cell model. In the present experiments, oxLDL had to be used in a higher concentration than acLDL in order to obtain cells with comparable amounts of total cholesterol after 24 h. Also, cholesterol esterification is higher in acLDL-laden cells. These two observations point to differences in uptake and intracellular processing of the modified lipoproteins. As total cell protein after lipid loading was not affected, this procedure proved to be non-toxic to the macrophages.

In this study NOS activity in J774 cells was induced by LPS. NOS metabolizes L-arginine to NO and citrulline, which both accumulate extracellularly. Nitric oxide spontaneously converts to the more stable products nitrite and nitrate, about 60% and 40% respectively, with no conversion from one product to the other.<sup>14</sup> Nitric oxide is presumably the main source of nitrite, whereas citrulline is involved in more complex biochemical pathways and can also be derived from L-arginine through the consecutive action of arginase and ornithine transcarbamylase. IFN- $\gamma$ , which on its own was a very weak stimulus for

NOS induction in J774 cells,<sup>3</sup> acted synergistically with LPS to induce higher NOS activity, as indicated by the increased release of nitrite and citrulline. This combination possibly also stimulated the arginase pathway, since the synergistic effect of IFN- $\gamma$  for citrulline release was much higher than for nitrite release. On the other hand, LPS plus IFN- $\gamma$  may have stimulated the macrophages for production of oxygen radicals, and reaction of superoxide anion and NO preferentially leads, via peroxynitrite, to nitrate,<sup>15</sup> at the expense of nitrite. In that case, citrulline is a better parameter of NOS activity than nitrite. Another explanation could be leakage of intracellular L-citrulline<sup>16</sup> in the supernatant, due to cell death. In view of these considerations, determination of both nitrite and citrulline in the cell-free supernatant seems to be required to evaluate induced NOS activity.

NOS activity was investigated in J774 foam cells. AcLDL-laden cells, unstimulated or stimulated, were not different from control cells with respect to either nitrite or citrulline release, indicating that lipid accumulation as such did not necessarily alter induced NOS activity. However, in oxLDL-laden cells, two changes were observed. First, unstimulated oxLDL-laden cells already displayed some induced NOS activity, whereas acLDL-laden cells or control cells did not. This small induction of NOS activity was apparently not the result of LPS contamination in the oxLDL preparation since addition of the LPS-binding antibiotic polymyxin B did not prevent it. Secondly, the subsequent stimulation of oxLDL-laden cells with LPS or LPS plus IFN- $\gamma$  resulted in lower amounts of released nitrite, compared to acLDL-laden cells or control cells. This is not due to trapping of NO or preferential conversion of NO to nitrate by the oxLDL, since citrulline decreased as well. It is not clear from the present experiments whether the induction of the NOS gene, the activity of the NOS enzyme or the availability of L-arginine is decreased. It has been reported that oxLDL inhibits other LPS inducible gene products such as TNF $\alpha$ ,<sup>17</sup> IL-1 $\alpha$  and  $\beta$ ,<sup>18</sup> IL-6<sup>18</sup> and PDGF.<sup>19,20</sup> In these studies, mRNAs were suppressed, suggesting that inhibition took place at the level of transcription or signal transduction. The observation that oxLDL inhibits the induction of several cytokines and NOS points to a more general mechanism of action. Since glucocorticoids also inhibit the induction of many LPS inducible genes,<sup>21</sup> it was speculated that a common mechanism might be responsible.

It could be that cholesterol oxidation products with glucocorticoid-like activity are formed during the oxidation of LDL. However, although the GC receptor antagonist mifepristone was clearly capable of partially reversing dexamethasone mediated NOS inhibition, it was unable to prevent the inhibitory action of oxLDL on NOS activity.

Other cellular events initiated by oxLDL may be involved. For instance, it has been reported that oxLDL increases arachidonate metabolism to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and PGI<sub>2</sub>,<sup>22</sup> and some of these products may inhibit NOS induction.<sup>23</sup>

TNF $\alpha$  has been reported to be an autocrine mediator in NOS induction in murine macrophages.<sup>24,25</sup> In oxLDL-treated cells, TNF $\alpha$  expression after stimulation is decreased,<sup>17</sup> which could lead to decreased NOS induction. However, oxLDL did not induce TNF $\alpha$  transcription<sup>17</sup> in unstimulated murine cells, whereas NOS induction was observed. Although TNF $\alpha$  expression and NO production can occur independently, the possibility that the oxLDL-mediated NOS inhibition is TNF-dependent remains open.

In conclusion, the study demonstrated that induction of NOS activity is specifically inhibited in oxLDL- and not in acLDL-laden foam cells. The mechanism of the observed inhibition is not clear at this moment, but a glucocorticoid-like action of oxLDL does not seem to be involved in view of the failure of the GCR antagonist to prevent the suppression.

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