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J. DULAK, M. POLUS, I. GUEVARA, J. HARTWICH, I. WYBRAŃSKA, R. KRZESZ, A. DEMBIŃSKA-KIEĆ

OXIDIZED LOW DENSITY LIPOPROTEIN INHIBITS INDUCIBLE NITRIC OXIDE SYNTHASE, GTP CYCLOHYDROLASE I AND TRANSFORMING GROWTH FACTOR β GENE EXPRESSION IN RAT MACROPHAGES

Department of Clinical Biochemistry, Collegium Medicum, Jagiellonian University, Kraków, Poland

Several studies have already demonstrated that oxidized- LDL decreases nitric oxide (NO) generation by cytokine-stimulated macrophages. However, the mechanisms of such an inhibition have not been yet elucidated. NO generation by inducible nitric oxide synthase (iNOS) is dependent on the presence of cofactors for NO generation, tetrathydrobiopterin (BH_4) among them. The NO generation by these cells is also regulated by some endogenous inhibitors, like TGF-β. Therefore, the aim of our recent study was to investigate the influence of ox-LDL on the expression of iNOS and GTP cyclohydrolase I (GTP-CH I), the key enzyme involved in the BH_4 synthesis as well as the ox-LDL effect on TGF-β expression in rat macrophages stimulated with IFNγ (250 U/ml) and LPS (500 ng/ml). Macrophages, activated in this way, express iNOS, GTP-CH I, and TGF-β mRNA. This expression was inhibited when the macrophages were preincubated for 24 hours with ox-LDL (100 μg/ml). Quantitative PCR revealed about 10-fold inhibition of iNOS gene expression by ox-LDL. As a consequence of down-regulation of iNOS and GTP-CH I genes, almost 3-fold diminished generation of NO_2^- by rat macrophages was observed. An inhibition of the TGFβ mRNA expression was also found.
Our studies indicate that decreased NO generation by ox-LDL treated macrophages may be the result of the diminished expression of both iNOS and GTP-CH I genes. This effect may be mediated by the activity of certain endogenous inhibitors of gene expression, however, our studies exclude the TGF-β as a candidate for this activity.

Key words: *LDL, inducible nitric oxide synthase, nitric oxide, GTP-cyclohydrolase I, TGFβ, atherosclerosis*

INTRODUCTION

Migration of monocytes and their accumulation at the sites of endothelial injury, in concert with platelet adhesion and aggregation, initiates the development of atherosclerotic plaque (for review see: 1). Activation of

monocytes results in their transformation to macrophages. These cells generate numerous mediators, including 15-lipooxygenase products, growth factors (like PDGF-B, FGF), cytokines (like IL-1β, TNFα), or chemokines (like IL-8 and MCP-1) (1). Macrophages, together with activated vascular smooth muscle cells (VSMC) and endothelial cells modify low density lipoproteins (LDL), what results in oxidized LDL generation (2, 3). Modified LDL are accumulated in macrophages and VSMC, and transforms them into lipid-loaded foam cells (1—3).

In healthy vessels nitric oxide (NO) generated by constitutive endothelial nitric oxide synthase (eNOS) prevents such undesired effects and LDL oxidation and uptake by cells of the arterial wall is inhibited by NO (4—7). However, increased local concentration of both native LDL and ox-LDL may result in the inhibition of this protective activity of NO, through the suppression of the eNOS expression, its enzymatic activity or by NO inactivation (8, 9, 10—17). It has been also demonstrated that ox-LDL effect on macrophages resulted in decreased NO generation by cytokine stimulated inducible NOS (iNOS) (18—20).

NO generation requires the activity of NOS, the optimal concentration of a substrate (L-arginine), and cofactors, such as heme, flavins, NADPH and tetrahydrobiopterin (BH_4) (21—23). BH_4 is synthesised from GTP, by means of several enzymes actions. The first key enzyme in this pathway is GTP cyclohydrolase I (GTP-CH I) (24). Therefore, it may be supposed that ox-LDL, which inhibits NO generation in macrophages, may also influence the synthesis of tetrahydrobiopterin.

Suppression of NO synthesis may also result from the activity of endogenous iNOS inhibitors. It was demonstrated that transforming growth factor β (TGF-β) inhibits iNOS expression in macrophages and VSMC at the level of transcription, mRNA stability, protein synthesis and protein activity (25—27). Thus, the observed down-regulation of iNOS in macrophages by ox-LDL may result from the increased generation of iNOS inhibitors, including TGFβ.

To elucidate some of the mechanisms which may lead to ox-LDL-related inhibition of NO generation, we decided to investigate the influence of ox-LDL on iNOS, GTP-CH I and TGF–β expression in rat peritoneal macrophages stimulated with IFNγ and LPS.

MATERIAL AND METHODS

Reagents

RPMI 1640 medium and foetal bovine serum were purchased from Gibco BRL (Warsaw, Poland); Total RNA Extraction Kit, Tth DNA Polymerase from Promega (Madison, USA), primers were synthesized by TIBMOLBIOL or ARK (Germany). The reagents for non-radioactive Northern blot hybridization were purchased from Boehringer (Mannheim, Germany), all other chemicals derived from Sigma (St. Louis, USA).

Isolation and modification of LDL

Native LDLs (n-LDL) (density 1.019–1.063 g/ml) were isolated from human plasma by sequential ultracentrifugation. Oxidized LDLs (ox-LDL) were prepared according to the established protocols (15,18) by incubation of n-LDLs (1 mg protein/ml) with $CuSO_4$ (5 μM) at 37°C for 24 hours, dialysed in the dark against 0.01 M phosphate buffer, pH 7.4, for 48 hours at 4°C. The ox-LDLs were sterilized by filtration (0.22 μm pores) and stored at 4°C. The extent of oxidation was estimated by the measurement of lipid peroxidation and expressed as the amount of malondialdehyde (MDA) per milligram of LDL protein. The average value of MDA was 22.38 ± 8.44 nmol per milligram protein of ox-LDL.

Cell culture

a) influence of ox-LDL on NO generation

Rat peritoneal macrophages were isolated 4 days after i.p. injection of 0.1% glycogen diluted in sterile 0.9% NaCl. Macrophages were laid in 35 mm wells at the concentration of $3—4 \times 10^6$/well in RPMI 1640 medium containing 5% fetal calf serum at 37°C in the atmosphere of 5% CO_2. Two hours later the medium was changed to remove non-adherent cells and the macrophages were cultured for three days. Afterwards, the medium was replaced with the fresh one and the cells were incubated without or in the presence of ox–LDL (100 μg protein/ml) for 24 hours. Then the medium was replaced again and macrophages were incubated with IFNγ (250U/ml) and LPS (500 ng/ml) with or without ox-LDL for the following 6 or 24 hours at 37°C. Concomittant incubation with IFNγ and LPS was performed in order to obtain the synergistic stimulation of iNOS expression and NO generation. IFNγ and LPS alone are not such effective activators as they are together (28).

The iNOS , GTP-CH I and TGF-β gene expression

Isolation of RNA.

Total cellular RNA was isolated from macrophages, according to Chomczynski's and Sacchi's method (29) using Total RNA Extraction Kit. The RNA concentration and purity were controlled spectrophotometrically by the optical density measured at 260/280nm. RNA was diluted in RNAse–free water and kept deep frozen (–70°C).

Primers

The iNOS–specific primers (see *Table 1*) were used to generate 384 base pairs (bp) product. The GTP-CH I product, amplified with primers for rat GTP-CH I (*Table 1*) was 175 bp long, while the RT-PCR with TGF-β specific-primers (*Table 1*) gave a product of 312 bp. Primers for the rat GAPDH (*Table 1*) (housekeeping, reference gene) were used to produce 452 bp product as a control of RNA isolation and amplification.

RT-PCR

The mRNA synthesis was studied by means of RT–PCR assay (reverse transcription-polymerase chain reaction) at 6 and 24 h after addition of IFNγ/LPS. RT–PCR was carried out on 100 ng total RNA using Tth DNA polymerase following the protocol recommended by the manufacturer. Briefly, a 20 minutes reverse transcription step was performed at 62.5°C

(1U Tth DNA Polymerase, 1 mM $MnCl_2$, 1 μM downstream (3') primer (*Table 1*). Afterwards, 20 μl of chelating buffer (750 μM EGTA, 0.5 U PrimeZyme, 2.5 mM $MgCl_2$, 250 nM upstream (5') primer (*Table 1*) were added and 30 cycles of PCR were performed (94°C/20 sek, 62.5°C/20 sek, 72°C/20 sec) in UNO Thermoblock (Biometra). PCR products were analyzed on ethidium bromide stained agarose gel electrophoresis.

Table 1. Sequence of primers used for iNOS, GTP-CHI, TGFβ and GAPDH RT-PCR

Primer	Sequence
iNOS 5'	5' — tgg ctt gcc cct gga agt ttc tc
iNOS 3'	5' — tgt ctc tgg gtc ctc tgg tca aa
GTP-CH I 5'	5' — cag att gca gtg gcc atc aca g
GTP-CH I 3'	5' — gag gaa ctc ctc ccg agt ctt tg
TGFβ 5'	5' — agt cac ccg cgt gct aat ggt g
TGFβ 3'	5' — tcc ttg gtt cag cca ctg ccg
GAPDH 5'	5' — acc aca gtc cat gcc atc ac
GAPDH 3'	5' — tcc acc acc ctg ttg ctg ta

Quantitative RT-PCR

This measurement of iNOS expression was performed by co-amplification of iNOS cDNA with a known amount of standard DNA. Standard DNA was designed as described previously (29). Standard has the same primer region as the amplified iNOS mRNA, therefore, the co-amplification of these two molecules with only one pair of primers was possible. From each experimental group, the same amounts of total RNA (100 ng) were reversely transcribed in four separate tubes, at conditions stated above. After RT step 0, 1, 10 or 100 fg of standard DNA were added to each of the four tubes, together with chelating buffer (see above). 30 cycles of PCR were then performed in conditions described above. Due to differences in length between iNOS (384 bp) and standard DNA (344 bp), the products were clearly separated during agarose electrophoresis.

The amount of 100 ng of total RNA was choosen, as it consistently gave the iNOS PCR product in a qualitative PCR. Thirty cycles of PCR were performed, as with such a schedule the amplification of a target sequence from 100 ng of total RNA has been demonstrated to be still within exponential phase (31, 32).

Northern blot hybridization for iNOS expression

The cDNA probe for mouse iNOS (kindly provided by Prof. Carl Nathan and Dr. Xiao Wen-Xie, USA) was labeled with digoxygenin by the random primer method using DIG DNA Labeling Kit and manufacturer's protocols with our modifications (33).

20 μ g of RNA was loaded into 1 % agarose/formaldehyde gel and electrophoresis was performed at 3V/cm, for about 4 h. The appereance of sharp 28S and 18S rRNA bands was regarded as the proof of RNA integrity and equal loading. Afterwards, the capillary Northern blot transfer to a nylon membrane was performed overnight in 20xSSC. The membranes were then washed in 5xSSC, air-dried and RNA was fixed to a membrane by UV-light crosslinking (3 min).

The hybridization with DIG-labeled probes was performed in a Micro—4 Hybridization oven (Hybaid). Membranes were prehybridized for 2 h at 42 °C in a high SDS-hybridization buffer (7% SDS, 5 × SSC, 1% Blocking reagent, 50% formamide, pH 7.0). Afterwards, the probes were

added to a hybridization buffer. Working concentrations of probes were established at 20—30ng/ml buffer. Hybridization was performed overnight at 42°C. On the next day the membranes were washed twice with 2 × SSC, 0.1 % SDS at room temperature, followed by twofold wash with 0.1 × SSC, 0.1% SDS at hybridization temperature and blocked with excess amounts of 1% blocking reagent. The immunological detection with alkaline-phosphatase-labeled anti-digoxygenin antibody (1:3000 to 1:5000 stock dilution in 1% blocking reagent) was carried out for 1.5 h at room temperature. The hybridization was revealed by overnight reaction with the alkaline phosphatase substrates, NBT/X-phosphate, diluted in a buffer (Tris-HCl 100 mM, NaCl 100 mM, $MgCl_2$ 50 mM, pH 9.5)

Measurement of NO generation

Nitrites (NO_2^-) accumulated in the culture media were determined by the Griess reaction, with our modification (34). Absorbance was measured at 550 nm.

Statistical methods

Data are presented as means ±SD. When appropriate, statistical evaluation was done with Mann-Whitney test. The value of $P < 0.05$ was accepted as being statistically significant.

RESULTS

iNOS, GTP-CH I and TGF-β expression in macrophages stimulated with IFNγ and LPS

The iNOS mRNA was not detected by Northern blot hybridization in rat peritoneal macrophages cultured for three days *in vitro* (*Fig. 1*). When such cells were stimulated with IFN γ or LPS, the slight induction of iNOS expression was observed (*Fig. 1b,c*), while the activation with IFNγ and LPS resulted in strong induction of iNOS expression (*Fig. 1,2*). The expression of iNOS 24 hours after IFNγ/LPS stimulation was higher than that at 6 hours (*Fig. 2*).

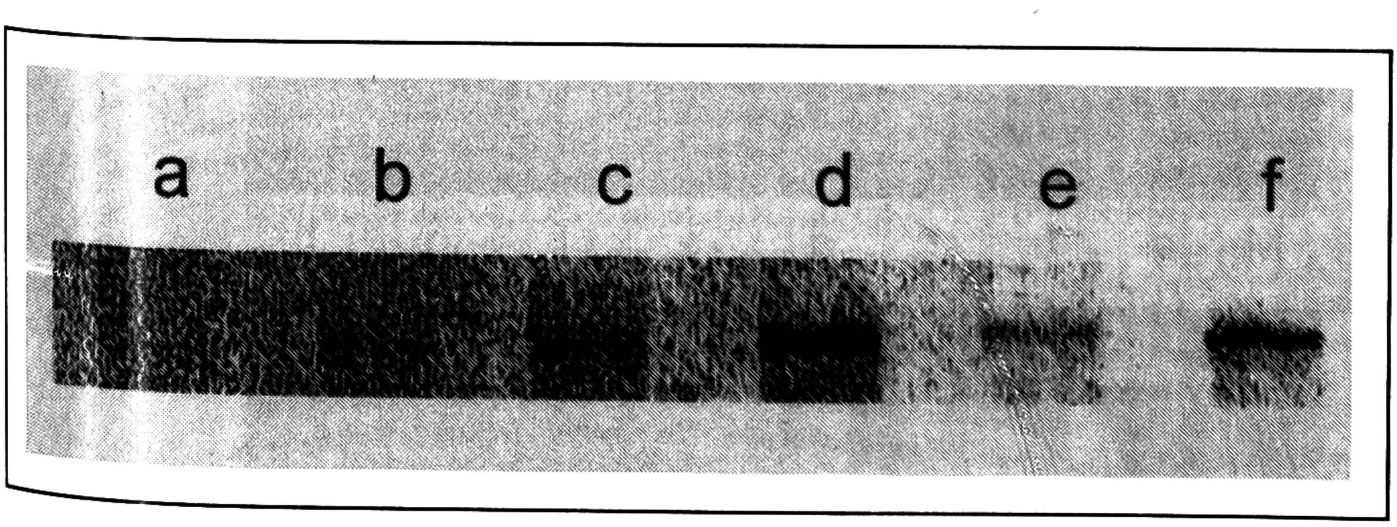

Fig. 1. Northern blot analysis of iNOS gene expression in rat macrophages a) control cells, b-f) cells stimulated for 6 h with: b) IFNγ (250 U/ml), c) LPS (500 ng/ml); d) IFNγ/LPS, e) ox-LDL (100 μg/ml), f) ox-LDL + IFNγ/LPS (for details see Materials and Methods).

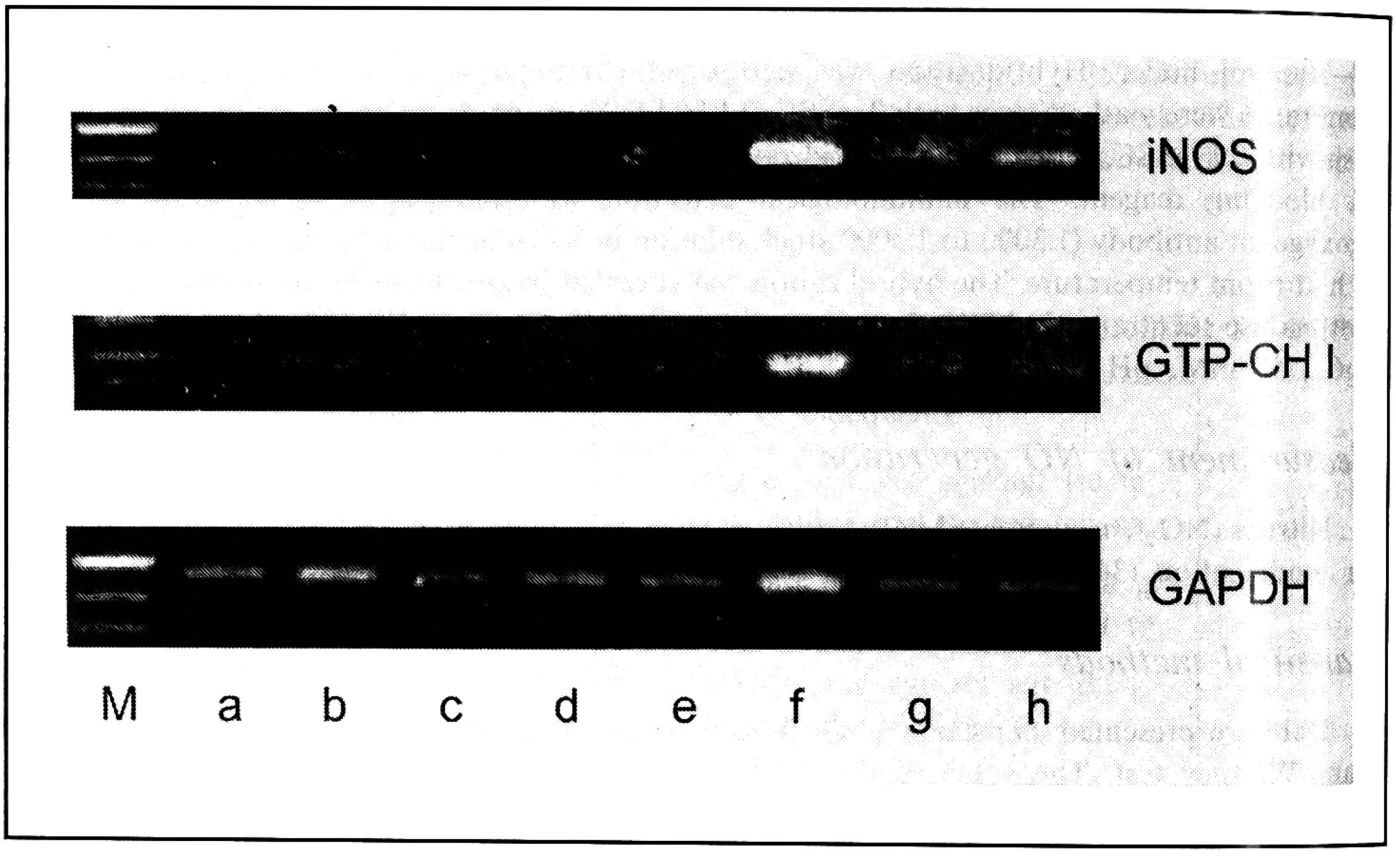

Fig. 2. Influence of ox-LDL on iNOS and GTP-CH I gene expression in rat macrophages — 6 h (a-d) and 24 h (e-h) after addition of IFNγ/LPS, a, e) control cells; b,f) IFNγ/LPS; c,g) ox-LDL; d,h) ox-LDL+IFNγ/LPS. Representative picture of three independent experiments. The increased expression of GAPDH visible in lane b and f resulted probably from the stimulation of this gene expression by NO, as has been already demonstrated (56).

Similarly to iNOS, the GTP-CH I gene expression was markedly induced after stimulation with IFNγ and LPS (*Fig. 2*), being higher after 24 hours than after 6 hours stimulation. The induction of TGF-β expression was also demonstrated in IFNγ/LPS stimulated cells (*Fig. 3*).

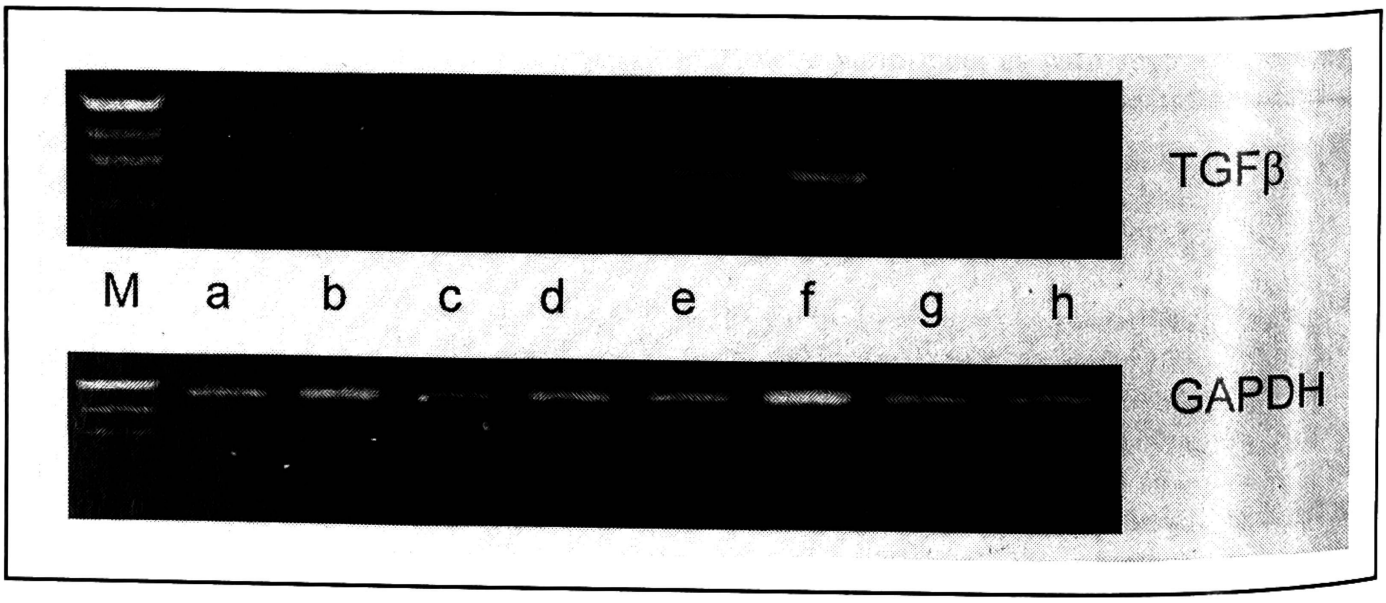

Fig. 3. Influence of ox-LDL on TGF-β gene expression in rat macrophages; a,e) control cells; b,f) IFNγ/LPS; c,g) ox-LDL; d,h) ox-LDL+IFNγ/LPS
Representative picture of three independent experiments.

Inhibition of iNOS, GTP-CH I and TGFβ expression by ox-LDL

Expression of iNOS, GTP-CH I and TGF-β was markedly down-regulated in macrophages pre-incubated with ox-LDL for 24 hours before stimulation with IFN-γ and LPS (*Fig. 1—3*).

In order to estimate the changes in the level of iNOS gene expression, the quantitative RT-PCR was performed (*Fig. 4*). In this experiment, the same aliquots of iNOS cDNA, synthesised by reverse transcription from iNOS mRNA, were amplified together with 0, 1, 10 or 100 fg of a standard DNA. Such a competitive PCR amplification results in the generation of iNOS and standard products, amounts of which are dependent on the starting concentration of iNOS cDNA and standard DNA. Lower starting concentration of iNOS cDNA than standard DNA resulted in greater amplification of standard DNA (e.g. *Fig. 4a*, lane 3 and 4) while the higher concentration of iNOS cDNA than standard DNA resulted in more iNOS than standard PCR product (e.g. *Fig. 4b* lane 1—3).

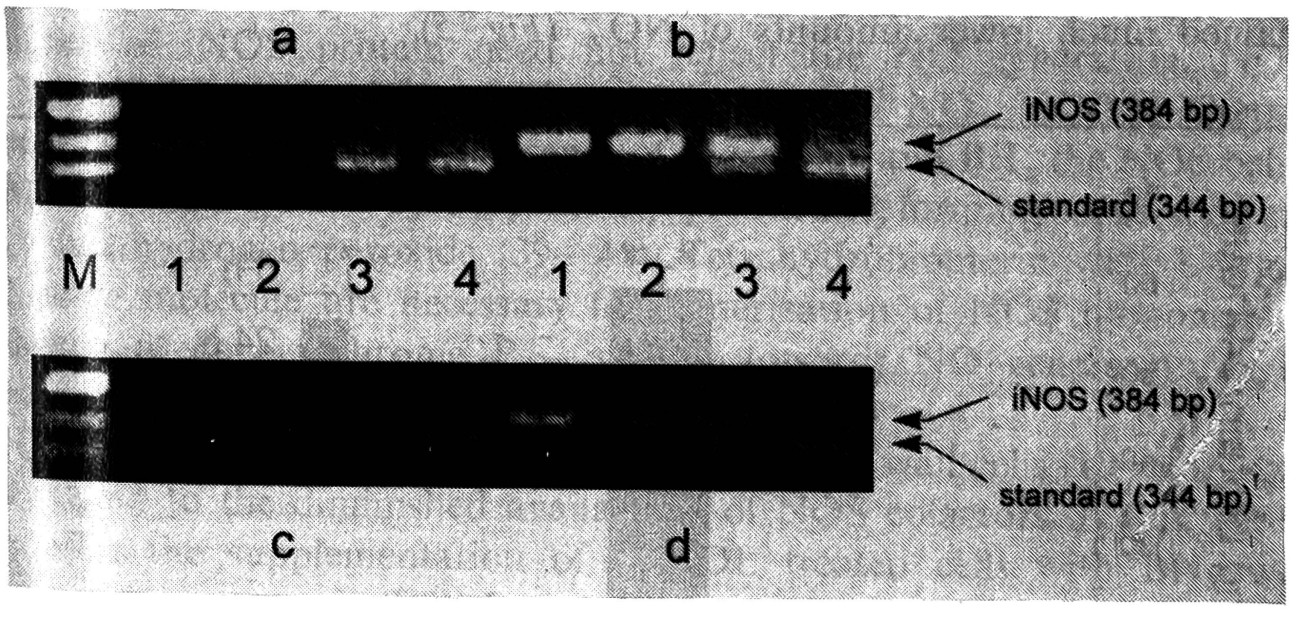

Fig. 4. Quantitative RT-PCR analysis of influence of ox-LDL on iNOS expression in rat macrophages, studied 6 h after IFN-γ/LPS stimulation

a) control; b) IFN-γ/LPS ; c) ox-LDL, d) ox-LDL+IFNγ/LPS

The same amount of total RNA (100 ng) from each group was reversely transcribed (RT) in four separate tubes (1—4 in each group); after RT the given amount of a standard DNA was added to the tubes as follows: 0 fg to tube 1; 1 fg to tube 2, 10 fg to tube 3; 100 fg to tube 4; and PCR was performed as described in Materials and Methods.

In control macrophages a weak iNOS PCR product was observed only in the absence of standard DNA or in the presence of 1 fg of standard (*Fig. 4a*, lane 1 and 2). The competitive amplification with larger amounts of standard molecules (10 or 100 fg) resulted in appearance of only standard PCR product (*Fig. 4a*, lane 3 and 4). Stimulation with IFNγ and LPS increased the amount

of iNOS PCR products to the amount comparable to 10—100 fg of standard DNA (*Fig. 4b*, lane 3 and 4), and reflected the results obtained by Northern blot hybridization (*Fig. 1*) or qualitative PCR (*Fig. 2*). In ox-LDL treated macrophages (*Fig. 4c*) the weak iNOS expression was observed similarly as in control cells (*Fig. 4a*). In macrophages pre-incubated with ox-LDL for 24 hours before IFNγ and LPS stimulation the iNOS expression was decreased about 10-fold, to the level comparable to 10 fg of standard DNA (*Fig. 4d*, lane 3).

NO generation

NO generation by rat macrophages was measured at 6 and 24 hours after addition of IFNγ and LPS. Stimulation with these compounds resulted in accumulation of NO_2^-, detectable with Griess' reaction after 24 hours but not yet at 6 hours after stimulation (*Fig. 5*). The amount of NO_2^- generated by IFNγ/LPS stimulated cells reached the value of 59.09 ± 2.0 μM/mg protein (*Fig. 5*). In contrast, cells which were preincubated with ox-LDL before addition of IFNγ and LPS generated about three times lower amounts of NO_2^- (*Fig. 5*). Medium from control cells and macrophages incubated with ox-LDL contained much lower amounts of NO_2^- (*Fig. 5*).

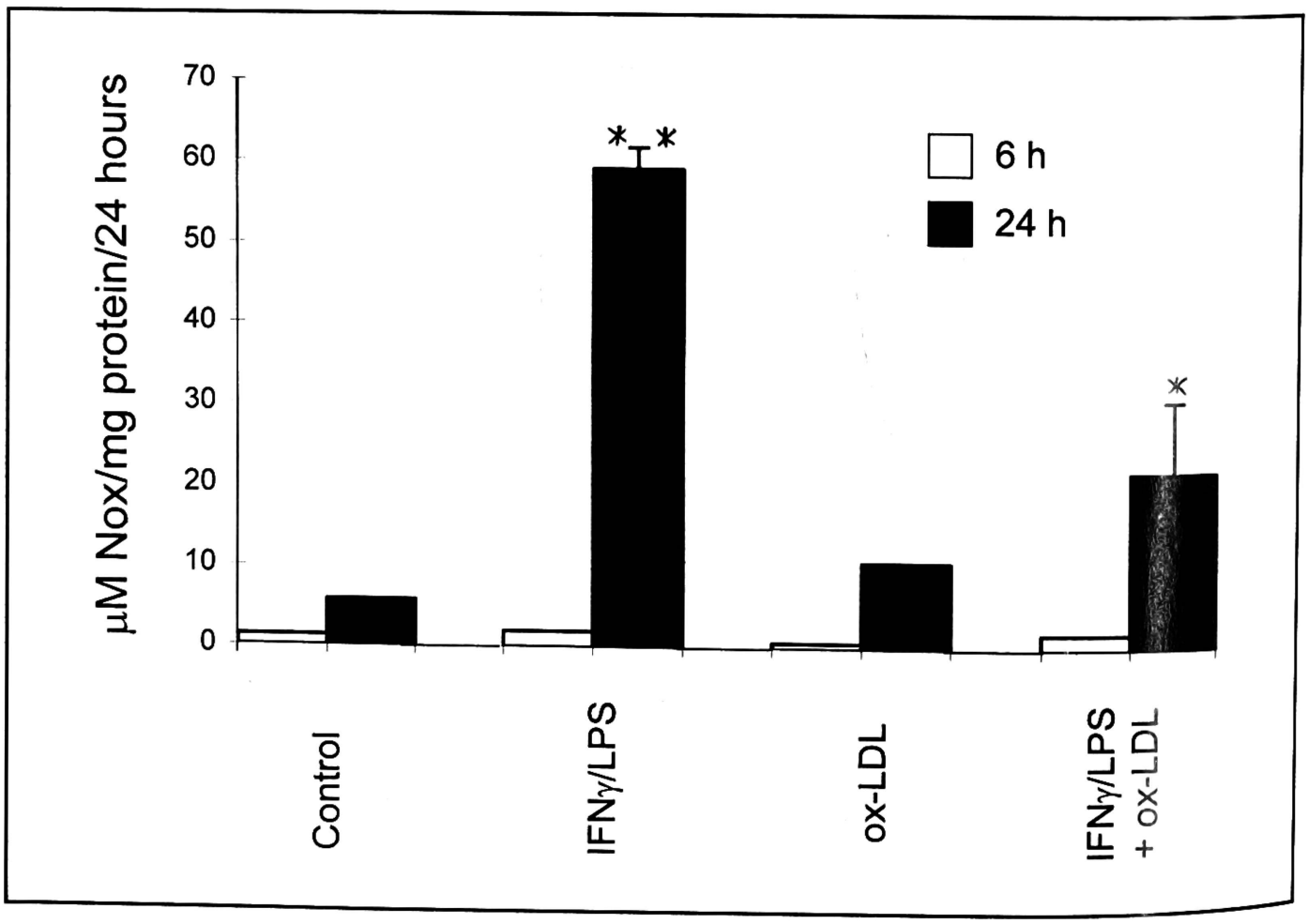

Fig. 5. Influence of ox-LDL on NO generation by rat macrophages studied 6 h (open bars) and 24 hours (filled bars) after stimulation with IFNγ/LPS. (mean ± SD; * $p. < 0.01$ vs IFNγ/LPS stimulation; ** $p. < 0.01$ vs control).

DISCUSSION

Numerous studies have demonstrated the inhibition of NO generation by ox-LDL in endothelial cells (10—15) and macrophages (18—20). These modified lipoproteins influence both iNOS gene expression, protein synthesis as well as bioavailability of NO. Increased generation of superoxide by endothelial cells in the presence of ox-LDL may be also responsible for inactivation of NO (16,17).

Our recent study has demonstrated that ox-LDL-mediated inhibition of NO generation by macrophages consists of some additional components. We have documented that ox-LDL inhibits not only iNOS expression in rat macrophages but also the induction of GTP-CH I gene expression, the key enzyme involved in the synthesis of tetrahydrobiopterin (BH_4) (22,23). Additionally, we demonstrated that ox-LDL impaired IFN/LPS mediated induction of TGF-β, a known inhibitor of iNOS in macrophages (25) and iNOS (26, 27) and GTP-CH I (35) in VSMC.

Several studies have demonstrated the iNOS gene expression in macrophages in the wall of the atherosclerotic vessels (36—38). However, the presence of iNOS protein need not reflect the NO generation. NOS is a NADPH-dependent dioxygenase generating NO by two separate monoxygenation steps. In the absence of L-arginine and BH_4 the NOS behaves as NADPH oxidase and cytochrome P_{450} reductase, forming superoxide anion (O_2^-) and hydrogen peroxide (39—41). Tetrahydrobiopterin with L-arginine and heme molecule are necessary for dimerization of NOS monomers and a change of its cytochrome P_{450} activity towards NO generation in such conditions is documented (22, 23, 40, 41).

Thus, diminished generation of NO by vascular wall cells (10—15) may be also related to the diminished availability of NOS cofactors. If this were the case, then the supplementation of ox-LDL treated cells with BH_4 would prevent this unwanted effect. In fact, studies on endothelial cells *in vitro* (42) and isolated rat aorta (43) proved that exogenous BH_4 can stimulate NO production in vascular endothelium. It was also recently demonstrated that infusion of BH_4 improved vasorelaxation in hyperlipidemic patients (44) and in our recent studies we have observed the BH_4-mediated restitution of NO generation in ox-LDL treated vascular smooth muscle cells (submitted).

TGFβ is one of the endogenous inhibitors of iNOS and GTP CH I gene expression and NO generation (25—27; 35). Therefore, we hypotesized that ox-LDL may exert its inhibitory effect on NO pathway *via* increased TGFβ synthesis. Our results suggest, however, that inhibition of iNOS in rat macrophages by ox-LDL cannot be related to TGF-β, as its expression induced by IFNγ and LPS was also inhibited by ox-LDL pre-treatment. It is not known whether ox-LDL inhibits TGFβ *in vivo*, but it has been recently

demonstrated that while active TGF-β is present in the serum of healthy individuals, its amounts are greatly diminished or absent in patients with atherosclerosis (45).

The iNOS expression in mouse macrophages stimulated with LPS and IFN-γ is regulated by several transcription factors. LPS stimulation results in activation of NF-κB factor (46), whereas IFN-γ requires IFN-regulatory element (IRF—1) (21). The rat iNOS gene promoter has been cloned and it has been proved to contain the NF-κB consensus sequence (47). Similarly to iNOS, the GTP-CH I transcription was reported to be regulated by NF-κB activity (48). Therefore, inhibition of NF-κB and/or IRF—1 transcription factors activity may result in the decrease in iNOS and GTP-CH I gene expression in rat macrophages.

A role of NF-κB in ox-LDL mediated inhibition of iNOS expression has been suggested in numerous studies. It has been previously shown that LPS-induced activation of NF-κB in human macrophages was substantially reduced after pre-incubation with ox-LDL (49, 50). Recently, Brand et al. (51) have demonstrated that prolonged (24 hours) incubation with ox-LDL inhibits the proteasome-mediated degradation of IκBα, the inhibitory protein preventing NF-κB binding to its region in gene promoter. Inhibition of NF-κB by stabilised Iκ-B may thus result in the lack of expression of NF-κB dependent genes.

The activity of the other transcription factors in the regulation of iNOS expression has recently been reported. The peroxisome proliferator associated receptor γ (PPARγ), which is expressed at the highest levels in adipose tissue and serves as a central regulator of adipocyte differentiation, is also involved in monocyte/macrophages differentiation and uptake of ox-LDL (52,53). It was demonstrated that activation of PPARγ inhibits the synthesis of inflammatory cytokines (54), the iNOS expression and NO generation (55) in macrophages. Thus, it is also possible that PPARγ activation by ox-LDL may inhibit the other genes of NO generating pathway, like, e.g. GTP-CH I.

In summary, our studies demonstrated the ox-LDL-mediated inhibition of iNOS, GTP-CH I and TGFβ gene expression in IFNγ/LPS stimulated rat peritoneal macrophages. Inhibition of GTP-CH I expression is a novel mechanism connected with the inhibitory activity of ox-LDL on NO generation. It may result in diminished synthesis and availability of BH_4 and lead to the increased superoxide formation by iNOS in atherosclerotic vessel wall.

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Author's address: Józef Dulak, Ph.D., Aldona Dembińska-Kieć M.D., Ph.D. Department of Clinical Biochemistry, Jagiellonian University, Collegium Medicum, Kopernika 15a, 31-501 Kraków, POLAND

fax: +48 12 421-40-73; phone: +48 12 421-40-06, email: mbdulak@cyf-kr.edu.pl

Dr. Jozef Dulak, Department of Cardiology, University of Innsbruck, Anichstrasse 35, A-6020 Innsbruck, Austria

fax: +43-512-504-3379; email: josef.dulak@uklibk.ac.at