0026-895X/98/061076-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**1076–1082 (1998).

Reactive Oxygen Species Regulate Macrophage Scavenger Receptor Type I, but Not Type II, in the Human Monocytic Cell Line THP-1

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Received August 22, 1997; Accepted February 13, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The uptake of modified low density lipoprotein via the macrophage scavenger receptor (MSR) results in the formation of lipid-laden foam cells during atherosclerosis. Because increased oxidative stress has been implicated in the pathogenesis of atherosclerosis, the role of reactive oxygen species on the activity and expression of MSR was investigated. The uptake of acetylated low density lipoprotein and the levels of MSR-I mRNA were inhibited by treatment with the oxygen radical scavengers 2,2,6,6-tetramethylpiperidine-*N*-oxyl, dimethylthiourea or sodium benzoate, or the iron chelator deferoxamine. Dimethylthiourea or benzoate also decreased the levels of MSR-I mRNA in the presence of the transcription inhibitor actinomycin D. These results indicate that hydroxyl radicals produced from superoxide anions and hydrogen peroxide in the presence of free iron, contribute to an increased MSR activity by stabilizing MSR-I mRNA. Several sources of reactive oxygen species are involved as inhibition of MSR activity and levels of MSR-I mRNA occurred in the presence of rotenone, a mitochondrial complex I inhibitor, or acetovanillone, a NADPH oxidase inhibitor. The (oxidative) stress responsive nuclear factor κB is not involved as inhibitors of its activation remained without significant inhibition. In contrast to MSR-I, the levels of MSR-II mRNA, which is formed by alternative splicing of the same gene transcript, were largely unaffected by the inhibitors of reactive oxygen species formation and activity. The present results suggest that oxidant stress contributes to an increased activity of MSR by stabilizing MSR-I mRNA.

The MSR mediates the uptake and degradation of polyanionic macromolecules including chemically modified proteins, such as oxidized LDL and AcLDL [for review, see Krieger (1992) and Wu *et al.* (1992)]. Degradation of modified LDL results in elevated intracellular cholesterol levels. Unlike the LDL receptor, expression of the MSR is not down-regulated by high levels of intracellular cholesterol. Therefore, excess supply of modified LDL will result in the intracellular formation of droplets containing cholesterol esters. The subendothelial formation of these lipid-laden foam cells from macrophages is an early event in the pathogenesis of atherosclerosis (Ross, 1993). Treatment of Watanabe heritable hyperlipidemic rabbits with dextran sulfate, an antagonist of the MSR, inhibited the formation of foam cells as well as atherosclerotic lesions (Tsubamoto *et al.*, 1994). In mice lacking the tumor necrosis factor receptor p55, MSR expression is increased and atherosclerosis accelerated (Schreyer *et al.*, 1996). Expression of MSR is present in atherosclerotic plaques and increased in hyperlipidemic patients (Matsumoto *et al.*, 1990; Villanova *et al.*, 1996). Therefore, investigation in the regulation of the expression of MSR may provide new insights for the treatment of atherosclerosis. The expression of MSR is low in circulating monocytes, but is substantially increased during their differentiation into macrophages (Geng *et al.*, 1994). Two subtypes of MSR exist that are produced by alternative splicing of a common gene transcript (Emi *et al.*, 1993). The first eight exons are shared by both subtypes and encodes the binding site. In addition, MSR-I has a extracellular cysteine-rich carboxyl-terminal domain encoded by exons 10 and 11, whereas MSR-II has a short 6-amino acid domain in its carboxyl-terminal end. The ratio between isoforms changes during differentiation

This work was supported by a fellowship from the Niels Stensen Foundation (the Netherlands) and the ONO Pharmaceutical company (Japan). S.J.D.K. receives a fellowship from the Netherlands Heart Foundation.

ABBREVIATIONS: MSR, macrophage scavenger receptor; LDL, low density lipoprotein; AcLDL, acetylated low density lipoprotein; ROS, reactive oxygen species; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; DMTU, dimethylthiourea; MTT, 3-[4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; PDTC, pyrrolidine dithiocarbamate; AP-1 activator protein-1; PMA, phorbol-12-myristate-13-acetate; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide.

from monocyte to macrophage because MSR-I is up-regulated (Geng *et al.*, 1994).

Several studies now suggest that increased expression of proteins, such as scavenger receptors, cytokines, inducible nitric oxide synthase, and cell adhesion molecules, is correlated in some way with increased levels of ROS and the oxidative modification of lipoproteins in the vessel wall (Lo *et al.*, 1993; Kiener *et al.*, 1995; Mietus-Snyder *et al.*, 1997). The importance of oxidant stress leading to endothelial injury and atherosclerosis has recently been reviewed by McGorisk and Treasure (1996). A relationship clearly exists between oxidative stress and the formation of ROS, resulting in the activation of proatherogenic redox-sensitive genes and the generation of mediators leading to atherosclerotic lesion formation. Several critical signaling processes seem to involve ROS. For example, stimulation of vascular smooth muscle by platelet-derived growth factor requires intracellular generation of hydrogen peroxide (Sundaresan *et al.*, 1995). Additionally, ROS mediate activation of the epidermal growth factor receptor induced by UV irradiation (Huang *et al.*, 1996) and cytokine-induced activation of c-*jun* amino-terminal kinases (Lo *et al.*, 1996). Therefore, ROS seem not only to be essential in signaling pathways maintaining cellular function, but also in cellular dysfunction leading to disease. We have investigated the role of ROS on the activity and expression of MSR in the human monocyte/macrophage cell line THP-1 differentiated by the phorbol ester PMA.

Materials and Methods

MSR activity. THP-1 cells were grown in suspension in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 μM β-mercaptoethanol, and 10% fetal bovine serum. All incubations were performed at 37° in a humidified atmosphere containing 5% CO_2. Cells were seeded onto 6-well plates at 1.5×10^6 cells per well. PMA (0.1 μM) was added to differentiate THP-1 cells into macrophage-like cells. Drugs were added 30 min before the addition of PMA. After 24 hr cells, which had adhered to the bottom of the well, were washed, lifted from the dish, and transferred to microcentrifuge tubes. After centrifugation (1600 $\times$ *g* for 3 min) the cells were incubated at 37° for 90 min in RPMI containing 30 μg/ml AcLDL labeled with DiI-AcLDL. Subsequently, cells were washed with phosphate-buffered saline and after centrifugation (1600 $\times$ *g* for 3 min) fixed in 1% paraformaldehyde in phosphate-buffered saline. To determine the fluorescence of the THP-1 cells, the suspension was measured by flow cytometry (FACScan, Becton Dickinson), and 10^4 cells were analyzed using the Lysis software (Hassall, 1992; Tsubamoto *et al.*, 1994). For every sample, the median of the fluorescence per cell was determined and expressed as percentage of cells treated with PMA alone.

Levels of MSR-I and II mRNA. The levels of mRNA for MSR were determined by the sensitive PCR after RT of total RNA (RT-PCR). THP-1 cells were cultured in T25 flasks at 4×10^6 cells in 4 ml. PMA (0.1 μM) was added to differentiate THP-1 cells into macrophage-like cells. Drugs were added 30 min before the addition of PMA. After 24 hr, cells that had adhered to the bottom of the flask were washed and resuspended in 1 ml of RNazol B (Biogensis, Poole, UK) to isolate total RNA. A pilot experiment showed that, at 24 hr, mRNA levels for MSRs were not different from those at 48 and 72 hr. Chloroform (0.1 ml) was added to separate RNA from DNA and proteins by extraction. The aqueous phase was collected, and isopropanol (1:1 v/v) was added to precipitate RNA, which, after centrifugation, was washed in 70% ethanol and dissolved in 1 mM EDTA.

GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA) was used to perform RT-PCR according to the instructions supplied. From 1 μg of total cellular RNA, cDNA was derived using random hexamers (2.5 μM) and 50 units of MulV RT in 20 μl at 42° for 15 min followed by denaturation at 99° for 5 min. Subsequently, cDNA was amplified by PCR using specific primer pairs (0.2 μM) and 2.5 units of AmpliTaq DNA polymerase in 100 μl during 35 cycles of denaturation at 91° for 1 min, annealing at 54° for 1 min, and extension at 72° for 2 min. The sense primer for type I and II MSR was selected from a sequence that is shared between the two subtypes: 5′-TGGGAACAT-TCTCAGACCTTGAG-3′. The antisense primer specific for the cysteine-rich domain of the type I MSR receptor was: 5′-TTGTC-CAAAGTGAGCTGCCTTGT-3′ (PCR product, 447 bp). The antisense primer for MSR-II was: 5′-TGCCCTAATATGATCAGTGAGTTG-3′ (PCR product, 291 bp). A primer set for PCR amplification of GAPDH was used as a control, sense primer: 5′-TGAAGGTCGGAGT-CAACGGA-3′ and antisense primer: 5′-GTGTCGCTGTTGAAGT-CAGA-3′ (PCR product, 858 bp). The PCR products were separated on a 1% agarose gel and after electrophoresis stained with ethidium bromide. The total amount of PCR product was quantified by scanning densitometry using the Bio-Rad (UK) Gel-doc system and expressed as percentage of THP-1 cells treated with PMA alone.

MSR mRNA stability. To investigate whether ROS influence the stability of MSR mRNA after the differentiation of THP-1 cells to macrophages, the influence of DMTU, sodium benzoate, and cycloheximide on mRNA levels was tested in the presence of the transcription inhibitor actinomycin D. THP-1 cells were cultured in 6-well plates at 1.5×10^6 cells in 2 ml. PMA (0.1 μM) was used to differentiate THP-1 cells into macrophage-like cells and, after 20 hr, actinomycin D (7.5 μg/ml) was added followed by the different drugs. After 3 and 7 hr, cells were homogenized in 1 ml of RNazol B, total RNA was isolated, and levels of MSR mRNA were determined by RT-PCR reaction as described above.

Adhesion and viability of THP-1 cells. Differentiation of THP-1 cells with PMA into macrophage-like cells results in the adhesion of cells to the bottom of the well and the expression of MSR. To assess the specificity of any drug effects on the expression or activity of MSR, adhesion (DNA content) and viability (MTT conversion) of the THP-1 cells was determined. THP-1 cells were seeded onto 96-well plates at 1×10^5 cells in 100 μl of medium per well. Drugs were added, and after 30 min the cells were differentiated using PMA (0.1 μM). After 24 hr, the wells were washed, and the adherent cells were solubilized by addition of water, shaking, and incubation at 37° for 1 hr. Hoechst 33258, a fluorescent dye for DNA, was added and incubated for another 20 min. Fluorescence was determined with excitation at 355 nm and emission at 460 nm. In another set, MTT was added to the cells (0.4 μg/ml final concentration) at 24 hr and incubated for 1 hr. In viable cells, MTT is converted to an insoluble purple formazan by dehydrogenase enzymes in the mitochondria. Subsequently, the wells were washed, and adherent cells were solubilized with dimethylsulfoxide. The absorbance of the converted dye was measured at 550 nm with background subtraction at 650 nm. In both tests, the result of adherent THP-1 cells treated with PMA alone was set at 100%.

Drugs. THP-1 cells were obtained from the European Type Culture Collection (Porton, UK). TEMPO and acetovanillone (apocynin) were purchased from Aldrich (Dorset, UK). Calpain inhibitor I was obtained from Calbiochem (Nottingham, UK). Enzymes and reagents for RT-PCR were purchased from Perkin-Elmer, except for the PCR primers, which were ordered from Severn Biotech Ltd. (Worcs, UK). DiI-AcLDL and RNazol B was obtained from Biogenesis (Poole, UK). All other drugs were purchased from Sigma (Dorset, UK).

Data analysis

All data are expressed as mean ± standard error of *n* observations. DiI-AcLDL internalization in THP-1 macrophages was determined in two separate samples per experiment. Viability and adherence of THP-1 cells was measured in triplicate, and the average was used in subsequent calculations. Statistical analysis was performed using one-way analysis of variance followed by Dunnet's test for multiple

comparison of separate treatments with control (THP-1 cells differentiated with PMA).

Results

MSR activity. The uptake of DiI-AcLDL by differentiated THP-1 cells was concentration-dependent, inhibited by excess unlabeled AcLDL, and prevented by incubation at 4° (Fig. 1). Differentiation of THP-1 resulted in a 5-fold increase in the uptake of DiI-AcLDL. The uptake was prevented by treatment with the protein synthesis inhibitor cycloheximide (1 μg/ml, 9 ± 4%, $n = 6$, $p < 0.01$). To investigate the role of ROS in the induction of the uptake of AcLDL, cells were treated with various oxygen radical scavengers (Fig. 2). TEMPO (1 mM), an intracellular scavenger of superoxide anion (Samuni *et al.*, 1990), DMTU (10 mM), an intracellular hydrogen peroxide scavenger (Parker *et al.*, 1985; Curtis *et al.*, 1988), and sodium benzoate (25 mM), a hydroxyl radical scavenger (Sagone *et al.*, 1980), inhibited the enhanced DiI-AcLDL uptake. The iron chelator deferoxamine (10 μM) also prevented the increase in DiI-AcLDL uptake. Furthermore, inhibition of ROS generation in the mitochondria by respira-

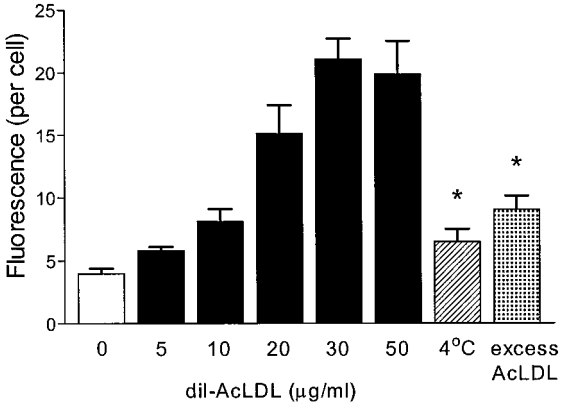

Fig. 1. The uptake of DiI-AcLDL in differentiated THP-1 macrophages. PMA (0.1 μM) was added to THP-1 cells, and after 24 hr macrophages were collected and incubated for 90 min with different concentrations of DiI-AcLDL at 37° (■), with 30 μg/ml DiI-AcLDL at 4° (▨) or with 30 μg/ml DiI-AcLDL in the present of excess unlabeled AcLDL (500 μg/ml, ▩). Subsequently, cells were analyzed by flow cytometry, and fluorescence (median) was determined. □, Autofluorescence of differentiated THP-1 macrophages. *Error bars*, mean ± standard error ($n = 6$). *, $p < 0.05$ compared with incubation with 30 μg/ml DiI-AcLDL alone.

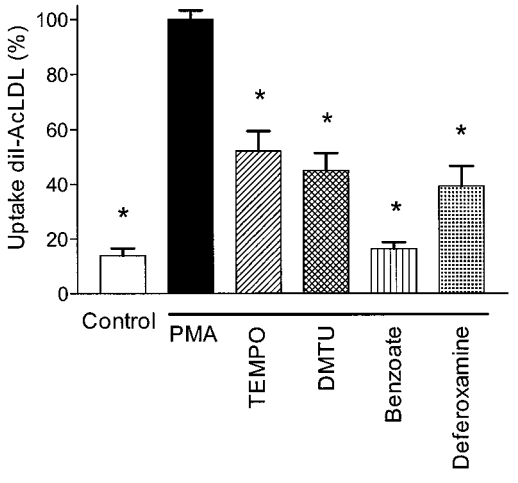

Fig. 2. ROS mediate the induction of DiI-AcLDL uptake in THP-1 macrophages. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of TEMPO (1 mM, ▨), DMTU (10 mM, ▩), sodium benzoate (25 mM, ▥), or deferoxamine (10 μM, ▤). After 24 hr, cells were incubated with 30 μg/ml DiI-AcLDL and analyzed by flow cytometry. □, Uptake of DiI-AcLDL by nondifferentiated THP-1 cells. Mean ± standard error are presented as percentage of PMA control ($n > 10$). *, $p < 0.05$ compared with PMA control.

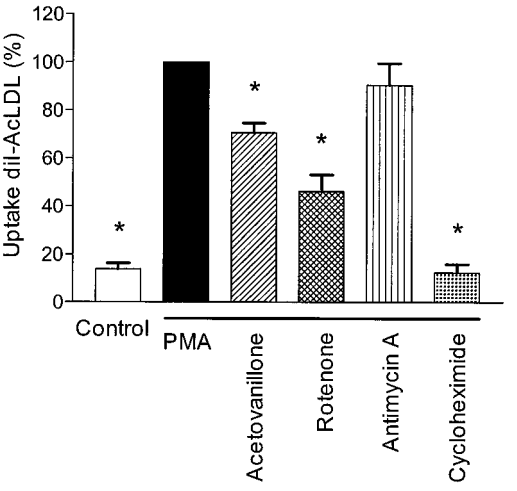

Fig. 3. The induction of DiI-AcLDL uptake in THP-1 macrophages requires the generation of ROS and protein synthesis. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of acetovanillone (50 μM, ▨), rotenone (1 μM, ▩), antimycin A (1 μM, ▥), or cycloheximide (1 μg/ml, ▤). After 24 hr, cells were incubated with 30 μg/ml DiI-AcLDL and analyzed by flow cytometry. □, Uptake of DiI-AcLDL by nondifferentiated THP-1 cells. Mean ± standard error are presented as percentage of PMA control ($n > 10$). *$p < 0.05$ compared with PMA control.

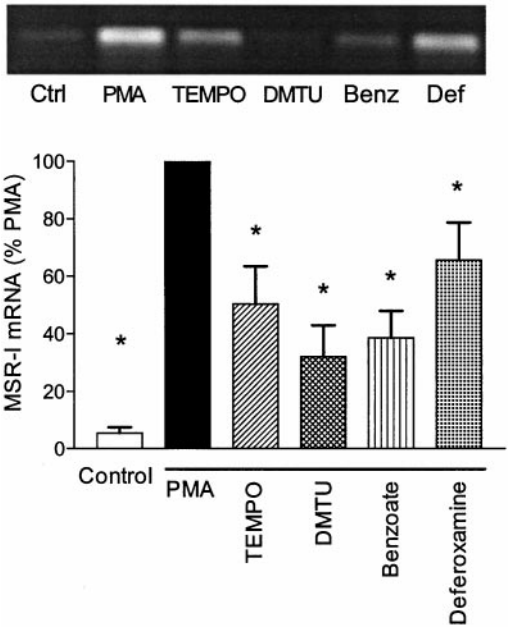

Fig. 4. ROS contribute to the increase in mRNA levels of MSR-I in THP-1 macrophages. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of TEMPO (1 mM, ▨), DMTU (10 mM, ▩), sodium benzoate (25 mM, *Benz*, ▥), or deferoxamine (10 μM, *Def*, ▤). After 24 hr, total RNA was isolated, and RT-PCR was performed with primers specific for MSR-I. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). □, mRNA levels of MSR-I mRNA in nondifferentiated THP-1 cells (*Ctrl*). Mean ± standard error are presented as percentage of PMA control ($n > 6$). *, $p < 0.05$ compared with PMA control.

tory chain complex I inhibitor rotenone (1 μM) attenuated the induction of DiI-AcLDL uptake (Fig. 3). The complex III inhibitor antimycin A (1 μM) had no significant effect. Acetovanillone (50 μM) (Stolk *et al.*, 1994), an inhibitor of NADPH oxidase, also reduced the uptake of DiI-AcLDL. Two inhibitors of the activation of the stress response nuclear factor κB (NFκB, (Schreck and Baeuerle, 1991; Miyamoto *et al.*, 1994), the radical scavenger and metal chelator, PDTC (25 μM), and the IκB protease inhibitor calpain inhibitor I (3 μM), did not significantly influence the uptake of DiI-AcLDL (92 $\pm$ 5%, $n = 9$ and 103 $\pm$ 14%, $n = 6$).

Levels of MSR-I and II mRNA. Similarly, the levels of mRNA for MSR-I increased approximately 20-fold after the exposure of THP-1 cells to PMA. The levels of mRNA for the MSR-II receptor showed only a 3-fold increase. The increase in the levels of mRNA for MSR-I was significantly inhibited by the oxygen radical scavengers, TEMPO, DMTU, and sodium benzoate, and the metal chelator deferoxamine (Fig. 4). Cycloheximide, acetovanillone, and rotenone, but not antimycin A, also inhibited the levels of MSR-I mRNA (Fig. 5). The mRNA levels were not significantly affected by treatment with PDTC (83 $\pm$ 10%, $n = 5$) or calpain inhibitor I (95 $\pm$ 34%, $n = 5$).

The increase in the levels of MSR-II mRNA was only moderately affected by the different treatments (Figs. 6 and 7). Treatment with TEMPO and sodium benzoate inhibited the levels of MSR-II mRNA by 20%, whereas this was around 60% for the MSR-I subtype. Cycloheximide, deferoxamine, rotenone, and acetovanillone inhibited the levels of MSR-I mRNA, but had no significant effect on the mRNA levels of the MSR-II subtype. None of the treatments had a significant effect on the levels of GAPDH mRNA (Table 1).

MSR mRNA stability. Post-treatment of differentiated THP-1 cells with DMTU, sodium benzoate, or cycloheximide together with the transcription inhibitor actinomycin D, resulted in a significant decrease in the levels of MSR-I mRNA at 7 hr, but not at 3 hr, compared with differentiated THP-1 cells receiving actinomycin D alone (Fig. 8). Again, the levels of MSR-II mRNA were not significantly affected by these post-treatments (Fig. 9).

Adhesion and viability of THP-1 cells. THP-1 cells grow in suspension and differentiate into macrophage-like cells in the presence of PMA, resulting in adhesion of most cells to the bottom of the well (82 $\pm$ 7% of total cells at 24 hr). Treatment with cycloheximide or rotenone partially reduced the adhesion of THP-1 cells elicited by PMA as measured by DNA content of the adherent cells (Table 1). None of the other drugs inhibited the adhesion. The viability of the adherent cells, as determined by the reduction of MTT, was not significantly affected by any of the treatments including cycloheximide or rotenone (Table 1).

Discussion

A number of studies have shown that an increase in expression of MSR mRNA and protein results in an elevated uptake and breakdown of modified LDL (Matsumoto *et al.*, 1990; Geng *et al.*, 1994; Wu *et al.*, 1994). The present data in differentiated THP-1 cells support this notion, because the AcLDL uptake was (i) associated with an increase in mRNA levels of the MSR-I and II and (ii) inhibited by the protein

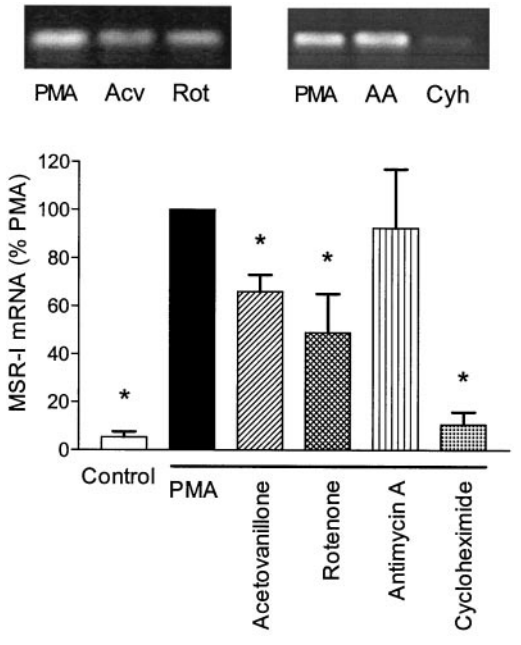

Fig. 5. The increase in mRNA levels of MSR-I in THP-1 macrophages requires the generation of ROS and protein synthesis. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of acetovanillone (50 μM, *Acv*, ▨), rotenone (1 μM, *Rot*, ▩), antimycin A (1 μM, *AA*, ▥) or cycloheximide (1 μg/ml, *Cyh*, ▤). After 24 hr, total RNA was isolated and RT-PCR was performed with primers specific for MSR-I. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). □, mRNA levels of MSR-I in nondifferentiated THP-1 cells (*Ctrl*). Mean $\pm$ standard error are presented as percentage of PMA control ($n > 6$). $*$, $p < 0.05$ compared with PMA control.

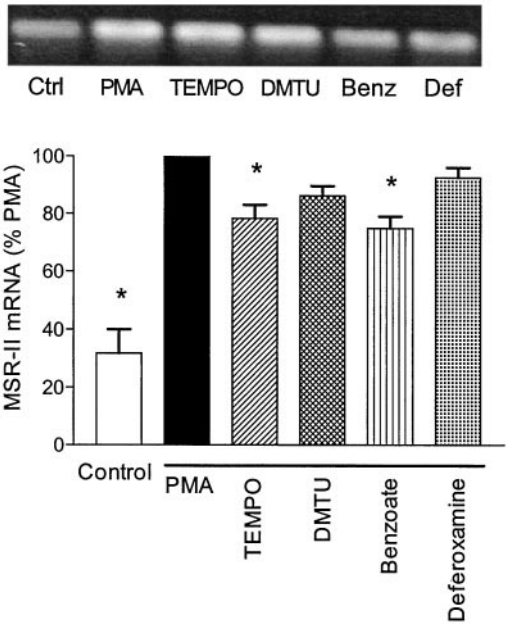

Fig. 6. Effect of scavengers of ROS on the mRNA levels of MSR-II in THP-1 macrophages. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of TEMPO (1 mM, ▨), DMTU (10 mM, ▩), sodium benzoate (25 mM, *Benz*, ▥) or deferoxamine (10 μM, *Def*, ▤). After 24 hr, total RNA was isolated and RT-PCR was performed with primers specific for MSR-II. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). □, Levels of MSR-II mRNA in nondifferentiated THP-1 cells (*Ctrl*). Mean $\pm$ standard error are presented as percentage of PMA control ($n > 6$). $*p < 0.05$ compared with PMA control.

synthesis inhibitor cycloheximide. Our results further indicate that ROS contribute to the increase in the uptake of AcLDL. Hydroxyl radicals are produced from superoxide anions and hydrogen peroxide in the presence of free iron via the Fenton reaction. Deferoxamine, an iron chelator, or scavengers of hydroxyl radicals (sodium benzoate), superoxide anions (TEMPO) and hydrogen peroxide (DMTU) inhibit the induction of AcLDL uptake. These results suggest an important role for hydroxyl radicals in modulation of MSR activity. Rotenone, but not antimycin A, decreased the uptake of AcLDL, which indicates that leakage of electrons in the mitochondria between enzyme complex I and III contributes to the generation of ROS-mediating MSR activity. Inhibition of ROS generation by NADPH oxidase by acetovanillone also decreased AcLDL uptake. Thus, several intracellular sources of ROS production contribute to the induction of MSR activity.

Recently, Mietus-Snyder *et al.* (1997) have demonstrated that scavenger receptor expression in smooth muscle cells elicited by phorbol ester treatment is mediated, in part, by ROS. They showed that after the activation of protein kinase C, intracellular levels of ROS rise, and that an elevated oxidative stress results in mRNA transcription and scavenger receptor activity. Here, the oxygen radical scavengers and inhibitors of ROS formation that attenuated MSR activity also decreased the levels of mRNA for the MSR-I receptor as detected by RT-PCR. This suggests that ROS regulate the uptake of AcLDL at the level of gene expression. Interestingly, the levels of mRNA for MSR-II was largely unaffected by the inhibitors of ROS formation or activity. As the two isoforms are produced by alternative splicing, the regulation of the respective isoform is post-transcriptional. This suggests that ROS affect MSR-I mRNA levels by a post-transcriptional mechanism. Indeed, post-treatment of differentiated THP-1 cells when transcription is blocked by actinomycin D, with the hydroxyl radical scavengers sodium benzoate and DMTU decreased the mRNA levels of MSR-I, but not MSR-II, when compared with differentiated cells receiving actinomycin D alone. The results indicate that ROS selectively contributes to the stabilization of MSR-I mRNA. Pre- and post-treatment with cycloheximide also attenuated the mRNA levels of MSR-I, but not the level of MSR-II.

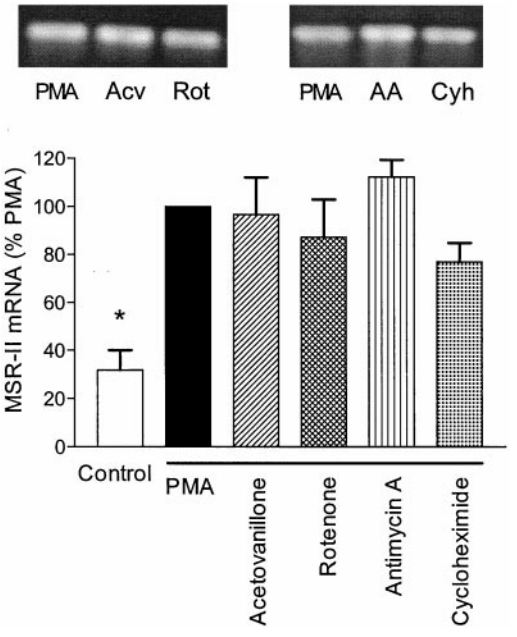

Fig. 7. The increase in mRNA levels for MSR-II in THP-1 macrophages is not dependent on protein synthesis or formation of ROS by NADPH oxidase or mitochondrial respiratory enzymes. THP-1 cells were differentiated by PMA (0.1 μM) in the absence (■) or presence of acetovanillone (50 μM, *Acv*, ▨), rotenone (1 μM, *Rot*, ▩), antimycin A (1 μM, *AA*, ▥), or cycloheximide (1 μg/ml, *Cyh*, ▤). After 24 hr, total RNA was isolated, and RT-PCR was performed with primers specific for MSR-II. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). □, Levels of MSR-II mRNA in nondifferentiated THP-1 cells. Mean ± standard error are presented as percentage of PMA control ($n > 6$). *, $p < 0.05$ compared with PMA control.

TABLE 1

Influence of treatments on adherence, viability and GAPDH mRNA in differentiated THP-1 cells

Data expressed as percentage of PMA control: mean ± SEM of 6–9 experiments.

Treatment	DNA	MTT/DNA	GAPDH
PMA control/THP-1 macrophages	100	100	100
+TEMPO (1 mM)	101 ± 4	101 ± 6	120 ± 24
+DMTU (10 mM)	84 ± 8	131 ± 23	123 ± 26
+Sodium benzoate (25 mM)	83 ± 5	107 ± 14	111 ± 19
+Deferoxamine (10 μM)	91 ± 7	92 ± 17	102 ± 19
+Acetovanillone (50 μM)	114 ± 23	100 ± 12	99 ± 11
+Rotenone (1 μM)	72 ± 8[a]	83 ± 8	91 ± 24
+Antimycin A (1 μM)	101 ± 7	102 ± 11	89 ± 11
+Cycloheximide (1 μg/ml)	57 ± 15[a]	132 ± 24	109 ± 34
+PDTC (25 μM)	97 ± 8	98 ± 8	114 ± 28
+Calpain inhibitor I (3 μM)	84 ± 7	108 ± 7	90 ± 13

[a] $p < 0.05$ compared with PMA control (analysis of variance, Dunnet's test).

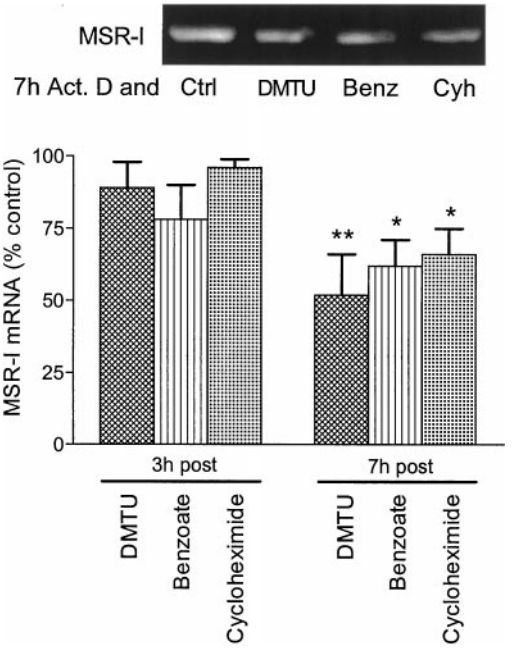

Fig. 8. ROS and protein synthesis contribute to the stabilization of MSR-I mRNA in THP-1 macrophages. THP-1 cells were differentiated by PMA (0.1 μM), and after 20 hr actinomycin D (7.5 μg/ml, *Act. D*) was added followed by DMTU (10 mM, ▩, $n = 5$), sodium benzoate (25 mM, *Benz*, ▥, $n = 5$), cycloheximide (1 μg/ml, *Cyh*, ▤, $n = 5$), or vehicle (*Ctrl*, RPMI medium, $n = 5$). After an additional 3 or 7 hr, total RNA was isolated, and RT-PCR was performed with primers specific for MSR-I. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). Mean ± standard error are presented as percentage of PMA-differentiated THP-1 macrophages treated with actinomycin D alone. *, $p < 0.05$ compared with PMA control.

Inhibition of MSR-I mRNA levels by cycloheximide has also been reported by Dufva *et al.* (1995). Thus, protein synthesis seems to be required for the stabilization of MSR-I mRNA. Another similarity is that post-treatment with cycloheximide or the scavengers DMTU and sodium benzoate was effective only after an incubation of 7 hr but not 3 hr. Possibly, ROS induce a protein that can selectively stabilize MSR-I mRNA by binding to a domain encoding for the cysteine-rich carboxyl terminus, which is lacking in the MSR-II mRNA.

The transcription factor NF-κB is oxidative stress sensitive (Schulze-Osthoff *et al.*, 1995; Sen and Packer, 1996), and the intracellular redox state of the cell seems to be crucial for its activation, which occurs concomitantly with a rise in ROS. However, neither PDTC or calpain inhibitor I, two unrelated inhibitors of the activation of NFκB, did significantly influence the levels of MSR mRNA or MSR activity. Therefore, other stress-responsive transcription factors than NFκB are involved in the induction of MSR expression and activity, and the post-transcriptional regulation of both subtypes. This may include AP-1 as ROS can up-regulate its activation (Schulze-Osthoff *et al.*, 1995; Lo *et al.*, 1996) and there seems to be a requirement for AP-1 expression in scavenger receptor constructs (Wu *et al.*, 1994).

In isolated human blood monocytes, the expression of MSR-I is similar or less to MSR-II (Geng *et al.*, 1994; Dufva *et al.*, 1995). During differentiation to macrophages, the expression of MSR increases, which is primarily attributed to an increase in the MSR-I isoform. This increase in MSR type I to type II ratio is maintained during the transformation of macrophages into foam cells (Geng *et al.*, 1994). Therefore, regulation of the MSR-I receptor may be important in the generation of foam cells and development of atherosclerotic plaques. In this study, differentiation of THP-1 cells to macrophages resulted in a 20-fold increase in the levels of MSR-I mRNA, but only in a 3-fold increase for isotype II. This further supports the notion that ROS and an as yet unknown protein contribute to a selective stabilization of MSR-I mRNA. Furthermore, the uptake of AcLDL is directly related to the selective influence of ROS inhibitors on MSR-I mRNA. Thus, THP-1 macrophages are useful model to study the regulation of the MSR receptors.

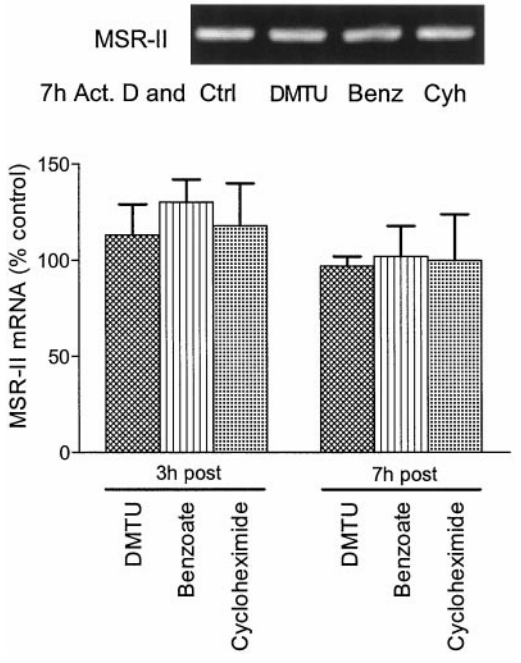

Fig. 9. ROS or protein synthesis do not contribute to the stabilization of MSR-II mRNA in THP-1 macrophages. THP-1 cells were differentiated by PMA (0.1 μM) and after 20 hr with actinomycin D (7.5 μg/ml, *Act. D*) was added followed by DMTU (10 mM, ▨, $n = 5$), sodium benzoate (25 mM, *Benz*, ▥, $n = 5$), cycloheximide (1 μg/ml, *Cyh*, ▧, $n = 5$), or vehicle (*Ctrl*, RPMI medium, $n = 5$). After an additional 3 or 7 hr, total RNA was isolated, and RT-PCR was performed with primers specific for MSR-II. After electrophoresis on a 1% agarose gel, PCR products were stained by ethidium bromide (a) and quantified by scanning densitometry (b). Mean ± standard error are presented as percentage of PMA-differentiated THP-1 macrophages treated with actinomycin D alone.

In macrophages, inhibition of MSR expression and activity has been reported in response to interferon-γ (Fong *et al.*, 1990; Geng and Hansson, 1992), transforming growth factor-β_1 (Bottalico *et al.*, 1991), tumor necrosis factor-α (Van Lenten and Fogelman, 1992; Hsu *et al.*, 1996), LPS (Van Lenten *et al.*, 1985), granulocyte/macrophage colony-stimulating factor (Van Der Kooij *et al.*, 1996), all-*trans*-retinoic acid, and dexamethasone (Moulton *et al.*, 1992). Tumor necrosis factor-α regulates MSR expression both by transcriptional and post-transcriptional mechanisms, but mainly by decreasing the half-life of both MSR-I and MSR-II mRNA via protein synthesis (Hsu *et al.*, 1996). The granulocyte/macrophage colony-stimulating factor also inhibits the expression of both isoforms (Van Der Kooij *et al.*, 1996). LPS inhibits MSR-I and MSR-II mRNA expression in human monocyte-derived macrophages to a different extent. Furthermore, the regulation of the expression of MSR-I, but not MSR-II, mRNA by LPS was cycloheximide sensitive. The present study shows that ROS mediates the stabilization of MSR-I, but not MSR-II, mRNA. Mietus-Snyder *et al.* (1997) have demonstrated that oxidative stress also results in an elevated mRNA. Thus, the regulation of MSR isoform expression is likely to be a complex transcriptional as well as post-transcriptional process involving ROS.

In conclusion, ROS contribute to an increase in the uptake of modified LDL in PMA differentiated THP-1 macrophages by stabilizing MSR-I mRNA. The rise in the levels of MSR-I mRNA involves post-transcriptional mechanisms independent of any regulation by NF-κB-like stress responsive transcription factors. However, other redox sensitive factors such as AP-1 seem to be functional in MSR regulation (Moulton *et al.*, 1992; Wu *et al.*, 1994). The present study suggests that during atherogenesis, oxidant stress may contribute to the formation of macrophage foam cells, not only by oxidizing LDL, but also by increasing MSR-I mRNA and activity.

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