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The Effects of Phosphoinositide/Calcium- or Cyclic AMP-mediated Signal Transduction Pathway Inhibitors on the Activation of Rat Peritoneal Macrophages by Acetylated Low-density Lipoprotein

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Abstract. *Background: Macrophages that uptake modified lipoproteins are activated and may initially behave as endotoxin-stimulated macrophages. This study was undertaken in order to determine whether signal transduction pathways controlling endotoxin-mediated activation may also influence the lipoprotein-mediated activation of macrophages. Materials and Methods: Rat peritoneal macrophages were incubated for 16 hours with acetylated low-density lipoprotein and certain agents that modify the phosphoinositide/calcium- and cyclic AMP-mediated pathways, such as 2-[4-morpholinyl]-8-phenyl-1[4H]-benzopyran-4-one (LY-294002), autocamtide 2-related inhibitory peptide (AIP), N-(2-[p-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89) and actinomycin D. The production of nitric oxide and the intracellular and extracellular activities of acid phosphatase were assayed. Results: Macrophages incubated with acetylated low-density lipoprotein showed an increased production of nitric oxide and intracellular acid phosphatase activity as compared to their controls. LY-294002, AIP and H-89 caused a significant decrease in nitric oxide production and intracellular acid phosphatase activity. Actinomycin D had similar effects. AIP and actinomycin also significantly increased extracellular acid phosphatase activity. Conclusion: The activation of peritoneal macrophages by acetylated low-density lipoprotein was similar to the activation by endotoxin, as expressed by the nitric oxide production and acid phosphatase intracellular activity; agents controlling the phosphoinositide/calcium- and cyclic AMP-mediated pathways in endotoxin-activated macrophages also influence the acetylated low-density lipoprotein-activated macrophages.*

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Atherosclerosis is a complex pathophysiological process in which modified lipoproteins are important participants. These lipoproteins are incorporated by macrophages, leading to the accumulation of esterified cholesterol and eventually to the formation of foam cells (1-4). The interaction of macrophages with lipoproteins affects the initiation, propagation and complication of the atherosclerotic lesion; the sequence of lipoprotein-triggered events is similar to that of inflammatory reactions and atherosclerosis is currently viewed by many as a form of inflammatory disease (5-9).

Macrophages may exist in three distinct functional states: quiescent, inflammatory or activated (10). Activation of mononuclear phagocytes by endotoxin (LPS) requires the interaction of several signal transduction pathways, including various kinases and phospholipases and the expression of many genes (11-13). When exposed to modified lipoproteins, macrophages acquire a phenotype similar to the LPS-induced activation (14-19), possibly due to the scavenger receptors binding these lipoproteins as well as inflammation- or infection-related ligands (20).

Macrophage activation can be regulated by cytokines, neuropeptides, hormones and autacoids (21-24). Macrophage activation is associated with increased production of hydrogen peroxide and increased activity of acid phosphatase, among other criteria (25). The production of hydrogen peroxide and the intracellular and extracellular activity of acid phosphatase were shown to be affected by agents modulating the cyclic AMP- protein kinase A and the phospholipase C - calcium - diacylglycerol - inositol phosphate signal transduction pathways (23, 24). In addition, these agents were shown to affect the production

of nitric oxide (Kondomerkos DJ, Kalamidas SA, Kotoulas OB, unpublished observations).

The aim of the present study was to determine: a) whether the activation of peritoneal macrophages by acetylated low-density lipoprotein (AcLDL) is similar to that obtained with LPS with respect to the production of nitric oxide and the activity of acid phosphatase, and b) whether signal transduction pathways controlling endotoxin-mediated activation may also influence the lipoprotein-mediated activation of macrophages. Thus, a) we determined the nitric oxide production and acid phosphatase activity in rat peritoneal macrophages activated *in vitro* by AcLDL, and b) we analyzed the effects of the specific inhibitors of phosphatidylinositol 3-kinase, 2-[4-morpholinyl]-8-phenyl-1[4H]-benzopyran-4-one (LY-294002), of calcium/calmodulin dependent II-kinase, autocamtide 2-related peptide (AIP), of cyclic AMP-dependent protein kinase, 2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89) and the inhibitor of protein synthesis actinomycin D on the nitric oxide production and acid phosphatase activity in rat peritoneal macrophages activated *in vitro* by AcLDL (26-28). Our results provide evidence that agents controlling the phosphoinositide/calcium- and cyclic AMP-mediated pathways also influence the AcLDL-activated macrophages.

Materials and Methods

Chemicals. Autocamtide 2-related inhibitory peptide (AIP, A-8348), Harris's hematoxylin solution (HHS-16), lipopolysaccharide (LPS, L-2880), low-density lipoprotein from human plasma (L-5402), 2-[4-morpholinyl]-8-phenyl-1[4H]-benzopyran-4-one (LY-294002, L-9908), N-(2-[p-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H-89, B-1427), N-(1-napthyl)-ethylene-diamine hydrochloride (N-5889), p-nitrophenyl phosphate disodium salt (N-4645) and sulfanilamide (S-9251) were obtained from Sigma (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). Acetic anhydride (45830) and Oil Red O (75087) were from Fluka (Fluka Chemie Gmbh, CH-9471, Buchs, Switzerland). Spectra/Por CE disposable dialyzers (135562, MWCO: 1000, diameter: 10mm) were from Spectrum (Spectrum Laboratories Inc., California, USA). Actinomycin D (10710) was from Serva (Serva, Heidelberg, Germany). β-glycerophosphate disodium salt (4168) and malachite green oxalate (1.01398) were obtained from Merck (Merck KGaA, Darmstadt, Germany). Sterile 24-well culture plates (143982) were manufactured by Nunc (Nalge Nunc International, Denmark). RPMI-1640 medium (F-1235) and L-glutamine (K-0282) were from Biochrom (Biochrom AG, Berlin, Germany). Stock solutions of Actinomycin D and LY-294002 were prepared in absolute ethanol. Low-density lipoprotein (LDL) was acetylated according to the original method by Basu *et al.* (29).

Animals and isolation of macrophages. Experiments were conducted with primary cultures of peritoneal macrophages. Cells were obtained as previously described (23, 24) from pathogen-free Wistar rats of both sexes, aged approximately 3 months; these were allowed *ad libitum* access to standard laboratory chow (pellets) and water. For each experiment, two rats of the same litter were used. The investigation and procedures conform to the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (30) and to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (31). Cells were suspended in cold phosphate-buffered saline and their density and viability were assessed on a Neubauer slide with trypan blue dye. Then they were resuspended in RPMI-1640 medium without fetal calf serum and plated into 24-well cell culture plates to achieve a final density of 5 x 10^5 cells per well (23, 24).

Cell culture. Each well was filled with RPMI-1640 medium supplemented with 20 mM HEPES, 100000 units/L penicillin, 100 mg/L streptomycin, 5.6 mg/L amphotericin-B and 0.3 g/L L-glutamine. Peritoneal macrophages were allowed to adhere to the wells during a 3-hour incubation at 37˚C in a CO_2-incubator (5% carbon dioxide - 95% air environment). Then, the wells were washed with warm phosphate-buffered saline and replenished with 1 ml medium. Phase-contrast observation with an inverted microscope revealed less than 2% contamination of the cell population with non-macrophage cells.

Macrophages in control wells were incubated for 16 hours with 50 μg/ml AcLDL whereas macrophages in experimental wells were incubated with 50 μg/ml AcLDL and the effector agent. The concentrations of these effector agents were: LY-294002, 30 μM; AIP, 12.5 μM; H-89, 60 μM; and actinomycin D, 8 μM. Each experiment was repeated twice, on different days. The results were pooled for statistical analysis. The "n" value (number of observations) in the Results section signifies the total number of control - experimental pairs included in the results.

Nitric oxide assay. Nitric oxide production during a 16-hour incubation period was estimated indirectly by assaying colorimetrically the accumulation of nitrite (32, 33). Macrophages were incubated in medium containing AcLDL and the effector agent. Then, the medium (1 ml) was aspirated from each cell well, centrifuged to sediment dislodged cells and transferred to test tubes containing 1 ml of Griess reagent. Absorbance was measured at 550 nm against a blank. The results are expressed as millimoles (mmoles) of nitrite±standard deviation produced per 5 x 10^5 cells.

Acid phosphatase activity determinations. Acid phosphatase activity was determined in both the supernatant (extracellular activity, exocytosed enzyme) and the cellular lysate (intracellular activity). The determinations were essentially as previously described (24) after a 16-hour incubation with Ac-LDL and the effector agent.

Briefly, for the extracellular activity, supernatants were aspirated from each well and replaced with 0.5 ml of serum-free RPMI-1640 medium and 0.5 ml of a 6 mg/ml solution of p-nitrophenyl phosphate in 0.1 M acetate buffer (final pH 5.2). The culture plates were incubated for 60 minutes in a CO_2-incubator. Then, the supernatant was transferred to 5 ml of 1N NaOH. Liberated chromogen was determined colorimetrically at 405 nm. For the intracellular activity (24), the culture medium was aspirated from the cell wells and these wells were washed with warm isotonic saline. To each well, 0.25 ml of cold lysis buffer was added and the plates were incubated for 30 minutes on ice. The lysates were diluted with 0.75 ml of warm 0.05 M acetate buffer and transferred to test tubes in a 37˚C water bath. To each tube, 1 ml of substrate

solution (20 mM sodium β-glycerophosphate and 8 mM EDTA in 50 mM acetate buffer, pH 5.2) was added. After a 60-minute incubation at 37˚C, the reaction was terminated by adding 0.095 ml of 70% perchloric acid and chilling in an ice-cold water bath for 15 minutes. Samples were centrifuged and 0.5 ml of malachite green color reagent (34) was added to the supernatant. The enzymatically released inorganic phosphorus was measured colorimetrically at 630 nm. The results were expressed as micrograms (μg) of phosphorus±standard deviation liberated per hour per 5 x 10^5 cells.

Light microscopy. To study morphological changes, macrophages were prepared as before (23, 24). The cells were cultured directly on sterile, acid-treated glass microscopy slides placed inside Petri dishes. After a 16-hour incubation with medium containing 50 μg/ml AcLDL, the slides were washed in warm phosphate-buffered saline and fixed in 4% phosphate-buffered formaldehyde. To evaluate foam cell formation, the slides were stained with Oil Red O (35). For the morphological evaluation, the slides were stained with Harris's hematoxylin. Specimens were studied with a Leitz Ortholux-II microscope, by two independent observers. Photographs were taken at a magnification of 1000, with a Leica DMLS microscope connected to a Sony Hi-Resolution CCD-IRIS Color Video Camera.

Statistical analysis. The pooled results from experiments conducted on different days were evaluated manually by the Student's (unpaired) *t*-test, according to Hill (36); *p* values smaller than 0.05 were considered as significant.

Results

In initial pilot experiments, 16-hour incubations of rat peritoneal macrophages were carried out with either 1 μg/ml LPS or with 50 μg/ml AcLDL. Macrophages incubated with LPS produced more nitric oxide than those incubated without LPS (controls: 0.055±0.004 mmoles nitrite / 5 x 10^5 cells, LPS-treated: 0.198±0.009 mmoles nitrite / 5 x 10^5 cells, n=9, $p<0.001$). Similarly, macrophages incubated with AcLDL produced more nitric oxide than those incubated without AcLDL (controls: 0.055±0.004 mmoles nitrite / 5 x 10^5 cells, AcLDL-treated: 0.158±0.009 mmoles nitrite / 5 x 10^5 cells, n=9, $p<0.001$). Macrophages incubated with LPS showed increased intracellular acid phosphatase activity as compared to their controls (controls: 2.377±0.221 μg/ml phosphate / hour / 5 x 10^5 cells, LPS-treated: 2.617±0.199 μg/ml phosphate / hour / 5 x 10^5 cells, n=10, $p<0.05$). Similarly, macrophages incubated with AcLDL showed increased acid phosphatase activity as compared to their controls (controls: 2.377±0.221 μg/ml phosphate / hour / 5 x 10^5 cells, AcLDL-treated: 3.064±0.287 μg/ml phosphate / hour / 5 x 10^5 cells, n=10, $p<0.005$).

Certain morphological changes produced by the addition of AcLDL to rat peritoneal macrophages were also observed. Cells incubated in culture medium without AcLDL usually appeared round or oval-shaped with a moderate amount of cytoplasm. Their size varied but very large cells were not common. Their nucleus was dark and usually round, oval with an indentation or kidney-shaped. Cells activated with 50 μg/ml AcLDL usually became quite large with abundant vacuolar cytoplasm and a large, lightly-stained, irregularly-contoured nucleus. Although occasional deposits were seen in the macrophages when stained with Oil Red O dye, no cells met the criteria for foam cells (35).

The incubation of peritoneal macrophages with AcLDL plus LY-294002 caused a significant decrease in nitric oxide production, as compared to cells treated with AcLDL only ($p<0.001$) (Table I). Treatment with AcLDL plus LY-294002 significantly decreased the intracellular ($p<0.001$), but did not affect the extracellular ($p>0.05$), activity of acid phosphatase (Table II).

The incubation of peritoneal macrophages with AcLDL plus AIP caused a significant decrease in nitric oxide production, as compared to cells treated with AcLDL only ($p<0.001$) (Table I). Treatment with AcLDL plus AIP significantly decreased the intracellular ($p<0.001$), but increased the extracellular ($p<0.001$), activity of acid phosphatase (Table II).

The incubation of peritoneal macrophages with AcLDL plus H-89 caused a significant decrease in nitric oxide production as compared to cells treated with AcLDL only ($p<0.001$) (Table III). Treatment with AcLDL plus H-89 markedly decreased the intracellular ($p<0.001$), but did not affect the extracellular ($p>0.05$), activity of acid phosphatase (Table IV).

The incubation of macrophages with AcLDL plus actinomycin D also decreased nitric oxide production ($p<0.001$) (Table III). Treatment with AcLDL plus actinomycin D decreased the intracellular ($p<0.001$), but increased the extracellular ($p<0.005$), activity of acid phosphatase (Table IV).

Discussion

The results of our study indicated that the activation of peritoneal macrophages by AcLDL, as expressed by the increased production of nitric oxide and activity of intracellular acid phosphatase, is similar to that obtained with LPS. The observed morphological changes also reflect the activation of the AcLDL-treated macrophages, as described before (23, 24, 37, 38). The effects of inhibitors used in our study suggested that the phosphoinositide/calcium and cyclic AMP-mediated signal transduction pathways might participate in the control of the AcLDL-induced activation of macrophages.

LPS-mediated nitric oxide production in macrophages is controlled either by phosphatidylinositol 3-kinase or by the protein kinase B - TOR (FRAP) pathway independently of phosphatidylinositol 3-kinase (39, 40). LY-294002, the inhibitor of phosphatidylinositol 3-kinase, moderates nitric oxide production in LPS- or interferon-activated macrophages

Table I. *The effects of LY-294002 or AIP on nitric oxide production in AcLDL-activated peritoneal macrophages.*

AcLDL	AcLDL + LY-294002	AcLDL + AIP
0.147±0.013 (14)	0.075±0.004 (14)	0.073±0.004 (14)

Results are expressed as mmoles nitrite±S.D. produced per 5 x 10^5 cells. Numbers in parentheses represent the number of observations (n) included in the results.

Table II. *The effects of LY-294002 or AIP on acid phosphatase activity in AcLDL-activated peritoneal macrophages.*

	AcLDL	AcLDL + LY-294002	AcLDL + AIP
Intracellular	2.605±0.111 (14)	2.324±0.082 (14)	2.024±0.096 (14)
Extracellular	1.267±0.046 (14)	1.242±0.034 (14)	1.480±0.055 (14)

Results are expressed as μg/ml inorganic phosphate±S.D. liberated per hour per 5 x 10^5 cells. Numbers in parentheses represent the number of observations (n) included in the results.

Table III. *The effects of H-89 or actinomycin D on nitric oxide production in AcLDL-activated peritoneal macrophages.*

AcLDL	AcLDL + H-89	AcLDL + actinomycin
0.114±0.011 (10)	0.033±0.004 (10)	0.058±0.009 (10)

Results are expressed as mmoles nitrite±S.D. produced per 5 x 10^5 cells. Numbers in parentheses represent the number of observations (n) included in the results.

Table IV. *The effects of H-89 or actinomycin D on acid phosphatase activity in AcLDL-activated peritoneal macrophages.*

	AcLDL	AcLDL + H-89	AcLDL + actinomycin
Intracellular	2.924±0.441 (10)	1.367±0.159 (10)	1.575±0.128 (10)
Extracellular	1.556±0.067 (10)	1.520±0.248 (10)	1.752±0.154 (10)

Results are expressed as μg/ml inorganic phosphate±S.D. liberated per hour per 5 x 10^5 cells. Numbers in parentheses represent the number of observations (n) included in the results.

(39-41). In the present study, the observed decrease of nitric oxide production from the AcLDL-activated macrophages after treatment with LY-294002 conforms with these findings. Phosphatidylinositol 3-kinase is related to the production of acid phosphatase, since LY-294002 was shown to induce reduction of tartrate-resistant acid phosphatase-positive osteoclasts (42). Phosphatidylinositol 3-kinase can exert regulatory functions on activated macrophages *via* nuclear factor kappa B and inhibition of the kinase leads to inhibition of the I-kappa B alpha degradation (40, 43). In the present study, the observed decrease in intracellular acid phosphatase activity after treatment with LY-294002 may also be related to the interaction of these signaling components.

Stimulation of macrophages with AcLDL leads to a sustained elevation of ionized calcium (44). Cytoplasmic ionized calcium levels participate in augmenting low nitric oxide production and hindering excessive nitric oxide production, these effects apparently independent of NFκB activation (45). Chelation of ionized calcium suppresses nitric oxide production and moderates macrophage immune capabilities (46). Some effects of calcium could be transduced *via* the calcium/calmodulin-dependent protein kinase II, a ubiquitous and multifunctional member of the calcium signaling pathway (47). In the present study, the decrease of nitric oxide production observed in AIP-treated macrophages complies with this possibility. Calmodulin and the calcium/calmodulin-dependent protein kinase II are also important signaling components in the process of phagosome-lysosome fusion; their inhibition contributes to the enhanced viability of microorganisms in the macrophages (48, 49). In the present study, the decrease in the intracellular activity of acid phosphatase observed in AIP-treated macrophages may be related to the inhibition of this function. In addition, the increase in the extracellular activity of acid phosphatase observed in AIP-treated macrophages may possibly be explained on the basis that, due to failure of the phagosome-lysosome fusion, this lysosomal enzyme is preferentially directed to the cell membrane, rather than to the phagosomes.

The cyclic AMP-protein kinase A pathway participates in LPS-induced macrophage activation (50, 51). It also increases the accumulation of cholesteryl esters derived from AcLDL lipoproteins in human macrophages (52). Cyclic AMP in LPS-activated macrophages modulates inducible nitric oxide synthase expression, moderating excessive and increasing low nitric oxide production (53-56). In the present study, the inhibitory action of H-89 is in accordance with these observations. Increased nitric oxide production effected by cyclic AMP-elevating agents in LPS-activated macrophages has also been found under our experimental conditions (Kondomerkos DJ, Kalamidas SA and Kotoulas OB, unpublished observations). Cyclic AMP

has been reported to decrease acid phosphatase production and secretion (57). The acid phosphatase activity of macrophages may be important in connection with the generation of free oxygen radicals (58-59). Our results with acid phosphatase activity in H-89-treated macrophages are difficult to explain. The intracellular activity may be lowered due to the inhibition of protein kinase A, which is important for macrophage activation (50, 51). This kinase may affect acid phosphatase production and activity at many levels, either directly or indirectly. Despite the fact that cyclic AMP is also an important regulator of exocytosis (60-62), no effect on the extracellular activity of acid phosphatase was noted in our experiments. Further studies are required to gain more insight into this issue.

The effect of actinomycin D on nitric oxide production and acid phosphatase intracellular activity undoubtedly reflects its inhibitory function on gene expression and protein synthesis. Actinomycin has been shown to inhibit LPS-induced production of nitric oxide in murine macrophages (63). The observation that the extracellular activity of acid phosphatase was increased by actinomycin cannot be explained by the data of this work, due to the wide range of genes and proteins affected by this protein synthesis inhibitor.

In conclusion, activation of rat peritoneal macrophages by acetylated low-density lipoprotein was similar to the activation by endotoxin, as expressed by the nitric oxide production and acid phosphatase intracellular activity. Agents controlling the phosphoinositide/calcium- and cyclic AMP-mediated pathways in endotoxin-activated macrophages also influence these pathways in acetylated low-density lipoprotein-activated macrophages.

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