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Application of multianalyte microphysiometry to characterize macrophage metabolic responses to oxidized LDL and effects of an apoA-1 mimetic

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ABSTRACT

Although the interaction of macrophages with oxidized low density liopoprotein (oxLDL) is critical to the pathogenesis of atherosclerosis, relatively little is known about their metabolic response to oxLDL. Our development of the multianalyte microphysiometer (MAMP) allows for simultaneous measurement of extracellular metabolic substrates and products in real-time. Here, we use the MAMP to study changes in the metabolic rates of RAW-264.7 cells undergoing respiratory burst in response to oxLDL. These studies indicate that short duration exposure of macrophages to oxLDL results in time-dependent increases in glucose and oxygen consumption and in lactate production and extracellular acidification rate. Since apolipoprotein A-I (apoA-I) and apoA-I mimetics prevent experimental atherosclerosis, we hypothesized that the metabolic response of the macrophage during respiratory burst can be modulated by apoA-I mimetics. We tested this hypothesis by examining the effects of the apoA-I peptide mimetic, L-4F, alone and complexed with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) on the macrophage metabolic response to oxLDL. L-4F and the DMPC/L-4F complexes attenuated the macrophage respiratory burst in response to oxLDL. The MAMP provides a novel approach for studying macrophage ligand–receptor interactions and cellular metabolism and our results provide new insights into the metabolic effects of oxLDL and mechanism of action of apoA-I mimetics.

1. Introduction

The mechanisms of atherosclerosis and its thrombotic complications remain incompletely understood. Considerable evidence supports the essential role of the monocyte/macrophage, oxidized low density lipoproteins (oxLDL), inflammation and both innate and adaptive immunity during all stages of atherogenesis [1,2]. Many of the biological effects of oxLDL are mediated by scavenger receptors expressed on the surface of monocytes/macrophages, vascular smooth muscle, and endothelial cells. In addition to type I and II class A scavenger receptors (SR-AI and SR-AII) which mediate oxLDL uptake and foam cell formation, other macrophage scavenger receptors that bind oxLDL include: CD36 (class B), CD68/macrosialin (class D) [3–5] and the lectin-like oxidized LDL receptor (LOX-1, class E) [5–7]. The biological activities of oxLDL are largely receptor mediated, thus attenuating ligand receptor binding and signaling might inhibit critical pathways involved in inflammation and atherosclerosis.

L-4F is an apoA-I mimetic peptide, which has been shown to enhance HDL anti-inflammatory function and to inhibit experimental atherosclerosis [8]. The sequestration of oxidized lipids by L-4F or some other modification of the physiochemical properties of oxLDL by L-4F could reduce the binding affinity/activity of oxLDL for macrophage scavenger receptor(s) and thereby inhibit foam cell formation. Very little is known about the metabolic consequences of oxLDL-scavenger receptor interactions. Understanding the metabolic pathways activated during atherogenesis should provide important new insights into the pathobiology of atherosclerosis and mode of action of apoA-I mimetics.

The multianalyte microphysiometer (MAMP) allows for continuous, real-time detection of acidification rate, glucose consumption, oxygen consumption, and lactate production. The MAMP has allowed determination of the metabolic effects of toxins [9–11], neural preconditioning [9,12], islets stimulation [13], and use of inhibitors to study pathways [14]. In the present study, we employed the MAMP to characterize the metabolic response of macrophages to oxLDL and also examined the effects of L-4F alone and complexed with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) on macrophage respiratory burst.

2. Materials and methods

All materials were used as obtained from the manufacturer unless otherwise noted. The macrophage cell line RAW-264.7

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was obtained from American Type Culture Collection (TIB-71, Manassas, VA). This cell line has been frequently used to study macrophage biology [15]. Culture media and supplements were obtained from the Media Core at Vanderbilt University (Nashville, TN) and Mediatech (Manassas, VA). Alamethicin was obtained from A.G. Scientific, Inc (San Diego, CA) and was reconstituted in 200 proof ethanol prior to use. Glucose oxidase (GOx, Type IIS from *Aspergillus niger*), bovine serum albumin (BSA, fraction V, 96%), and glutaraldehyde (glutaric dialdehyde, 25 wt.% solution in water) were purchased from Sigma. Stabilized lactate oxidase (LOx) was purchased from Applied Enzyme Technology (Pontypool, UK). Native human LDL was obtained from Intracel (RP-031, Frederick, MD). Human oxLDL, prepared by oxidation with $CuSO_4$, was obtained from Intracel (RP-047, Frederick, MD). OxLDL was mixed with running media (modified RMPI 1640, 5 mM glucose) to a final concentration of 50 μg/mL prior to exposure. Novartis Institutes for Biomedical Research, (Cambridge, MA) provided L-4F, which was reconstituted in water. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids, Inc. (Atlanta, GA). The DMPC/L-4F complexes were prepared according to published methods by Ota and coworkers [16]. Cytosensor® consumables were purchased from Molecular Devices Corporation (Sunnyvale, CA).

Experiments were performed using a Cytosensor® Microphysiometer coupled with a VIIBRE Multi-Channel Potentiostat (Vanderbilt Institute of Integrative Biosystems Research and Education, Nashville, TN). Cells were immobilized in sensor cups on the Cytosensor®, which controlled temperature, pump speed, and collected acidification data through light addressable potentiometric sensors (LAPS) [17–20]. LabVIEW programming enabled electrochemical measurements of additional analytes through the modified amperometric sensor current response, where the electrodes were held at a +0.6 V vs. Ag/AgCl (2 M KCl) potential. For electrochemical studies of oxygen consumption, the oxygen electrode was held at a potential of −0.45 V vs. Ag/AgCl (2 M KCl).

Cells were cultured in Dulbecco's Modified Eagle Medium, 50 mL FBS, 5.0 mL Sodium Pyruvate, 0.5 mL of an antifungal. The cells adhered to culture flasks and regularly split according to ATCC guidelines (www.atcc.org). RAW 264.7 cells were seeded into Corning Costar® Transwell® cell culture inserts (PTFE, 3 μm pores) at 2.5×10^5 cells per insert (RAW 264.7, CRL-2278 ATCC). After seeding onto the inserts, cells were incubated overnight at a constant 37 °C with 5% CO_2, using the aforementioned culture medium. After adhesion, cells were then sandwiched within the capsule using a spacer and insert, allowing for a chamber of approximately 3 μL in volume. Modified RPMI 1640 with additional glucose (5 mM) was utilized in each experiment at a perfusion rate of 0.1 mL/min. The flow cycle was 120 s:80 s flow period followed by 40 s of stop flow. The stop flow enabled an accumulation or consumption of analytes within the microfluidic chamber, detectible by the Cytosensor® and VIIBRE potentiostat.

For preincubation studies, cells were primed with either 20 μg/mL L-4F or 20 μg/mL DMPC/L-4F 12 h prior to oxLDL exposure. Extracellular acidification rates, glucose and oxygen consumption, and lactate production were measured over the course of one hour to obtain basal metabolic measurements before cellular exposure to 50 μg protein/mL oxLDL or to 50 μg protein/mL native LDL, which elicited maximum increases in responses [21]. After a 6 min exposure and 60 min recovery, the cells were killed using 85 μM alamethicin dissolved in RPMI. In the absence of cellular metabolic activity, sensor calibrations were then performed. Both glucose and lactate sensors were calibrated using a four step calibration: 0 mM glucose, 0 mM lactate; 1.5 mM glucose, 0.05 mM lactate; 3 mM glucose, 0.1 mM lactate; 5 mM glucose, 0.2 mM lactate. Following calibrations, a baseline was reestablished using 5 mM glucose running media.

OxLDL was preincubated with either 20 μg/mL L-4F or 20 μg/mL DMPC/L-4F for 120 min prior to exposing macrophages to the mixture for 6 min. The concentration of L-4F was chosen to be in molar excess (5–10-fold) of oxidized phospholipids based on measured binding affinities [8]. Metabolic parameters were measured during the subsequent 60 min recovery period using running media. These studies assessed whether L-4F or DMPC/L-4F could modify oxLDL in a manner which alters macrophage metabolic responses.

Acidification rates were determined by the Cytosoft program as described by Owicki [19,20]. The current response resulting from cellular activity at each time point, Δi_p, was calculated as the difference between the response of live cells and the average response in the chamber after the cells were killed by alamethicin, taking into account any mass transfer effects within the microfluidic chamber. Calibration curves were made for glucose and lactate with the dead cell current measurements. Oxygen concentration was determined with the baseline concentration of dissolved oxygen calibrated as 0.24 mM [22]. The resulting rates for glucose, lactate, and oxygen were then normalized against 5 initial basal time points 10 min prior to oxLDL exposure and plotted against time to show the dynamic metabolic time course [23]. The statistical significance of the effects of oxLDL on metabolic responses and of experimental interventions was determined by comparing MAMP mean peak height responses using a Student's paired or unpaired *t*-test as appropriate. A *p*-value of less than 0.05 was taken as statistically significant.

3. Results and discussion

Upon exposure of cells to 50 μg/mL oxLDL for 6 min, macrophage metabolism was stimulated as evidenced by an increase in extracellular acidification (Fig. 1). This was accompanied by increases in glucose consumption, lactate production, and oxygen consumption. These data indicate that oxLDL exposure promotes a respiratory burst response through both aerobic and anaerobic pathways.

In contrast to oxLDL, exposure of cells to 50 μg/mL of native (non-oxidized) LDL for 6 min resulted in only a small increase in glucose consumption (peak height increase of 14.0 ± 1.0%, *p* value of 0.037 vs mean basal rate), with no significant changes in lactate production, oxygen consumption or extracellular acidification.

Preincubation of oxLDL with L-4F for 120 min prevented the macrophage aerobic response and attenuated the anaerobic response to oxLDL (Fig. 2, Table 1). Exposure to oxLDL resulted in a

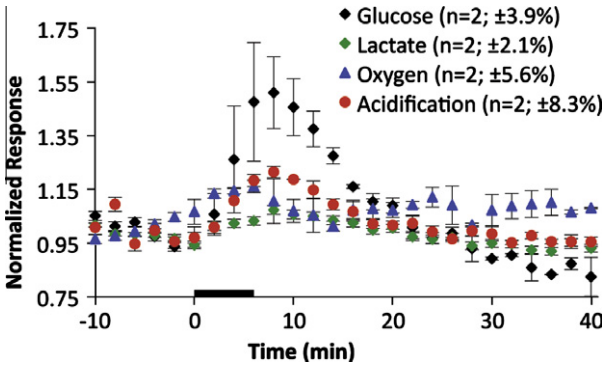

Fig. 1. Average metabolic response of RAW 264.7 cells to oxLDL. The black bar indicates the 6 min exposure to 50 μg/mL oxLDL. The number of experiments and standard error of the mean for the basal metabolic rate 10 min before exposure to ox-LDL are given in parentheses. Compared to mean basal metabolic rate, maximum MAMP peak height changes in glucose and oxygen consumption, lactate production and acidification rate were statistically significant at the $p < 0.05$ level.

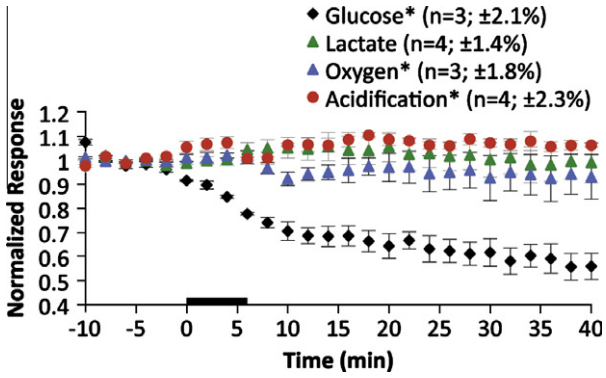

Fig. 2. Average metabolic response of RAW 264.7 cells to 50 μg/mL oxLDL (black bar) after 120 min preincubation of oxLDL with L-4F. The number of experiments and standard error of the mean for the basal metabolic rate 10 min before exposure to ox-LDL are given in parentheses. Compared to mean basal metabolic rate, maximum MAMP peak height changes in glucose and oxygen consumption and acidification rate were statistically significant at the $p < 0.05$ level.

Table 1
Comparison of effects of preincubating oxLDLwith L-4F or DMPC/L-4F on metabolic responses to oxLDL. Mean peak MAMP height changes and standard errors for each experimental condition are shown.

Peak height changes	Glucose	Lactate	Oxygen	Acidification
oxLDL alone	51.0 ± 13.4%	9.0 ± 1.6%	18.0 ± 2.6%	21.5 ± 2.2%
oxLDL preincubated with L-4F	−22.3 ± 0.5%*	5.6 ± 6.0%	−7.8 ± 2.8%*	10.3 ± 1.4%*
oxLDL preincubated with DMPC/L-4F	0.28 ± 6.8%*,**	1.1 ± 3.8%	3.3 ± 6.8%	12.2 ± 6.5%

* $p < 0.05$ vs oxLDL alone.
** $p < 0.05$ vs oxLDL precincubated with L-4F.

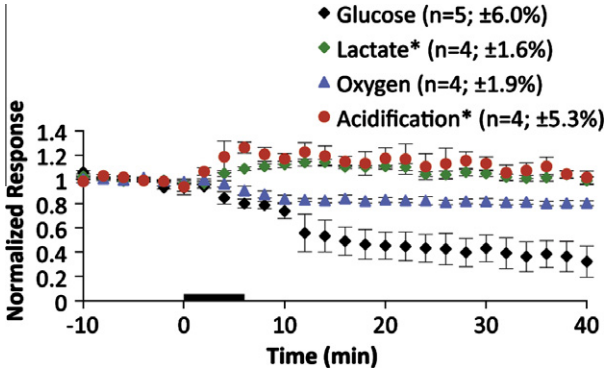

Fig. 3. Average metabolic response of RAW 264.7 cells to 50 μg/mL oxLDL (black bar) after 12 h preincubation of cells with 20 μg/mL L-4F. The number of experiments and standard error of the mean for the basal metabolic rate 10 min before exposure to ox-LDL are given in parentheses. Compared to mean basal metabolic rate, maximum MAMP peak height changes in glucose and oxygen consumption, lactate production and acidification rate were statistically significant at the $p < 0.05$ level.

smaller change in extracellular acidification, a reduction in glucose consumption, and a small reduction in oxygen consumption. Thus, L-4F attenuated the increase in macrophage acidification rate and lactate production in response to oxLDL while it also decreased glucose and oxygen consumption. These results indicate that short-term exposure of oxLDL to L-4F prevents the macrophage respiratory burst by inhibiting the aerobic response to oxLDL and also attenuating the anaerobic response.

Following preincubation of oxLDL with DMPC/L-4F complexes, the metabolic response of macrophages to oxLDL was characterized by a 12.2 ± 6.5% increase in extracellular acidification with no change in glucose or oxygen consumption or in lactate production (Table 1). Maximum metabolic responses (peak MAMP height changes) to oxLDL alone and to oxLDL following preincubation (120 min) with either L-4F or DMPC/L-4F complexes are summarized and compared in Table 1.

An additional experiment determined the effects of DMPC alone on macrophage respiratory burst. Phorbol 12-myristate 13-acetate (PMA) is known to initiate respiratory burst in macrophages. To assess the effect of DMPC on PMA induced respiratory burst, we exposed macrophages to a media containing both DMPC and PMA. Upon exposure to the DMPC/PMA mixture, macrophages showed an immediate increase in glucose consumption, lactate production, and extracellular acidification similar to that observed with PMA alone (data not shown). This indicates that the effects of DMPC on oxLDL induced macrophage metabolism are not the result of nonselective effects on respiratory burst.

Experiments were performed to determine if the metabolic response of macrophages to oxLDL is altered by preincubation of the cells with L-4F. The macrophage metabolic response to oxLDL following 12 h preincubation with 20 μg/mL L-4F is shown in Fig. 3. OxLDL stimulated macrophage extracellular acidification; the magnitude of effect was comparable to the change observed in response to oxLDL in the absence of L-4F (Fig. 1). Although the acidification response to oxLDL was not affected, pre-exposure of cells to L-4F reduced oxLDL induced glucose consumption without changing oxygen consumption, and increased lactate production.

This metabolic pattern contrasts with that observed in response to oxLDL without L-4F preincubation in which a comparable increase in acidification rate was accompanied by increases in both glucose and oxygen consumption and an increase in lactate production (Table 2). Thus, preincubation of cells with L-4F altered the macrophage metabolic response to oxLDL. The observed decrease in glucose and oxygen consumption without a change in extracellular acidification rate indicates that L-4F attenuated macrophage aerobic respiration by inhibiting respiratory burst, while increasing anaerobic respiration indicative of uncoupling of metabolic pathways.

Experiments were performed to determine if the metabolic response to oxLDL is altered by preincubation of the macrophages with DMPC/L-4F complexes. Results are shown in Table 2. The macrophage metabolic response to oxLDL following preincubation with 20 μg/mL DMPC/L-4F resulted in an increase in extracellular acidification rate. Changes in glucose and oxygen consumption as well as lactate production were comparable to the results obtained during preincubation with L-4F alone.

4. Conclusion

In the present study we employed multianalyte microphysiometery to characterize the metabolic response of murine macrophages to oxLDL and to examine the effects of an apoA-I mimetic on this metabolic response. Our results indicate that short-term exposure to oxLDL activates both aerobic and anaerobic pathways in the macrophage and that L-4F inhibits the response to oxLDL, preventing respiratory burst. These results provide new insights into the macrophage metabolic response to oxLDL and suggest a new mechanism of action for the anti-atherosclerotic effects of apoA-I mimetics.

De Vries and coworkers [21] were the first to show that oxLDL can acutely activate the metabolic state of macrophages by a receptor-mediated process by employing microphysiometry to measure changes in extracellular acidification. Our extracellular acidification data confirm the observations of De Vries, and also indicate that oxLDL increases glucose and oxygen consumption

Table 2
Comparison of effects of preincubating RAW264.7 cells with L-4F or DMPC/L-4F on metabolic responses to oxLDL. Mean peak MAMP height changes following exposure to oxLDL and standard errors are indicated for each experimental condition.

Peak height changes	Glucose	Lactate	Oxygen	Acidification
Cells with no preincubation	51.0 ± 13.4%	9.0 ± 1.6%	18.0 ± 2.6%	21.5 ± 2.2%
Cells preincubated with L-4F	−6.0 ± 1.3%*	14.0 ± 3.0%	−0.5 ± 0.8%*	26.1 ± 4.6%
Cells preincubated with DMPC/L-4F	17.3 ± 42.9%	10.9 ± 18.3%	−8.9 ± 18.8%	49.0 ± 26.3%

* $p < 0.05$ vs. cells with no preincubation.

and lactate production, indicating that oxLDL stimulates both aerobic and anaerobic respiration and induces respiratory burst.

Pre-incubation of oxLDL with L-4F attenuated the metabolic response of murine macrophages to oxLDL. Compared to the metabolic response to oxLDL, L-4F preincubation decreased oxLDL induced metabolic activation. Similarly, DMPC/L-4F also attenuated oxLDL-induced stimulation of extracellular acidification. One possible mechanism for these observations is that L-4F and DMPC/L-4F reduced ligand binding affinity/activity of oxLDL for one or more of its scavenger receptors. This could be due to modification of oxLDL, direct effects on scavenger receptors or both. ApoA-I peptide mimetics and L-4F in particular have been shown to bind oxidized phospholipids with much higher affinity than non-oxidized lipids or fatty acids [24]. It is also possible that L-4F and DMPC/L-4F alter the binding activity of oxLDL for the macrophage receptor(s) by absorbing lysophosphatidylcholine (lysoPC) from oxLDL. Ota et al. provided direct evidence for transfer of lysoPC from oxLDL to the phospholipid bilayers of DMPC/apoA-I, but not to apoA-I or DMPC alone [16]. LysoPC has been shown to act as an important mediator of the atherogenic effects of oxLDL [21,25–27]. Like oxLDL, lysoPC stimulates macrophage metabolism, suggesting that the metabolic effects of oxLDL may be mediated, in part, by lysoPC [28]. Finally, oxLDL could also be modified by incorporating L-4F into oxLDL. Transfer of apoA-I to oxLDL incubated with DMPC/apoA-I or apoA-I alone has been shown to occur [29].

Pre-incubation of macrophages with L-4F decreased glucose and oxygen consumption without a change in acidification rate. These results indicate that L-4F attenuated the macrophage metabolic response to oxLDL by decreasing aerobic respiration and inhibiting the respiratory burst. The altered macrophage metabolic response to oxLDL following pre-incubation of cells with L-4F also suggests the possibility that apoA-I mimetics could modulate the effects of oxLDL by direct effects on scavenger receptors. Since L-4F was present in the microphysiometer chamber during exposure to oxLDL, the effects could also be explained by rapid physiochemical modification of oxLDL with reduced binding affinity for scavenger receptors.

The inhibition of oxLDL induced macrophage aerobic respiration and prevention of respiratory burst by L-4F may have implications for the potential utility of apoA-I mimetic therapeutics for atherosclerotic cardiovascular disease. Since macrophage mediated oxidative stress is implicated in the pathogenesis of atherosclerosis, our results suggest a novel mechanism by which apoA-1 and apoA-1 mimetics prevent experimental atherosclerosis [31–33] and may also promote plaque stabilization by inhibiting metabolic activation of macrophages and respiratory burst. In a recent study, patients with coronary heart disease, L-4F administered by once daily intravenous infusion (7 days) or subcutaneous injection (28 days) showed no beneficial effects on selected plasma markers of HDL function or on hsCRP or IL-6 [30]. Plasma L-4F exposures were similar to those shown to reduce the HDL inflammatory index ex vivo and to also inhibit atherosclerosis in mice [31,32]. Our results suggest that another mechanism for the anti-inflammatory and anti-atherosclerotic actions of L-4F and perhaps other apoA-I mimetics in experimental models is their ability to inhibit macrophage metabolic activation in response to oxLDL, which could be independent of HDL functional effects.

The results of the present study showed that oxLDL stimulated metabolism of macrophages, particularly glucose uptake, which was inhibited by preincubation of oxLDL or macrophages with L-4F. These observations may have important diagnostic implications. Fluorodeoxyglucose-positron emission tomography (FDG-PET) has been thought to provide a measure of the severity of atherosclerotic plaque inflammation and cardiovasculasr risk [36]. Glucose uptake by metabolically active macrophages provides the basis for FDG-PET imaging. Tawakol et al. demonstrated a significant correlation between the PET signal from carotid plaques and macrophage content from corresponding histologic sections, establishing that FDG-PET imaging can be used to assess the severity of plaque inflammation in patients [34]. Studies in both animal models [35] and in humans [36] indicate that FDG-PET could be used to assess therapeutic efficacy of drugs that reduce plaque inflammation. Our observation that L-4F reduces metabolic activity and inhibits glucose consumption of oxLDL stimulated macrophages suggests that the FDG-PET signal may be attenuated not only by reducing the number of metabolically active macrophages in a plaque, but also by reducing the metabolic rate and respiratory burst of macrophages. Therapeutic implications of attenuating macrophage metabolism are uncertain as most anti-inflammatory agents affect inflammatory cell recruitment. It is possible that drugs targeting macrophage metabolism and oxidative stress pathways may help promote plaque stability and prevent rupture.

In summary, the metabolic response of RAW 264.7 cells to oxLDL was characterized using the MAMP to simultaneously measure acidification rate, glucose and oxygen consumption, and lactate production. OxLDL produced an increase in glucose and oxygen consumption, lactate production, and an increase in extracellular acidification indicating activation of both aerobic and anaerobic metabolic pathways. The attenuation of oxLDL induced aerobic respiration and respiratory burst by L-4F suggests a novel mechanism for the observed beneficial effects of apoA-I mimetics on development and progression of atherosclerosis by inhibiting oxLDL induced macrophage metabolic activation.

Acknowledgments

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