Local non-esterified fatty acids correlate with inflammation in atheroma plaques of patients with type 2 diabetes.

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Submitted 10 June 2009 and accepted 18 February 2010.

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Background: Atherosclerosis is prevalent in diabetic patients, but there is little information on the localization of non-esterified fatty acids (NEFA) within the plaque and their relationship with inflammation.

Objective: To characterize the NEFA composition and location in human diabetic atheroma plaques by metabolomic analysis and imaging, and address their relationship with inflammation activity.

Methods: Time-of-flight-Secondary ion Mass Spectrometry (TOF-SIMS) was used for metabolomic analysis imaging of frozen carotid atheroma plaques. Carotid endarterectomy specimens were used for conventional immunohistochemistry, laser-capture microdissection-quantitative PCR and in situ southwestern hybridization. Biological actions of linoleic acid were studied in cultured vascular smooth muscle cells (VSMC).

Results: TOF-SIMS Imaging evidenced a significant increase in the quantity of several NEFA in diabetic versus non-diabetic atheroma plaques. Higher levels of NEFA were also found in diabetic sera. The presence of lipoprotein lipase mRNA in NEFA-rich areas of the atheroma plaque, and the lack of correlation between serum and plaque NEFA, suggests a local origin for plaque NEFA. The pattern of distribution of plaque NEFA is similar to that of MCP-1, LPL and activated NF-kB. Diabetic endarterectomy specimens showed higher numbers of infiltrating macrophages and T lymphocytes, a finding which associated with higher NEFA levels. Finally linoleic acid activates NF-kB and up-regulates NF-kB-mediated lipoprotein lipase and MCP-1 expression in cultured VSMC. Conclusion: There is an increased presence of NEFA in diabetic plaque neointima. NEFA levels are higher in diabetic atheroma plaques than in non-diabetics. We hypothesize that NEFA may be produced locally and contribute to local inflammation.
Atherosclerosis is the major cause of death among diabetic patients, accounting for 50% of their mortality (1). Diabetes-associated atherosclerosis has been estimated to affect 5 to 8% of the general population and is by itself a major cause of death and disability in developed countries. Many factors have been postulated to link both conditions. Among these factors we find the proinflammatory and cytotoxic actions of high glucose levels and the generation of advanced glycation end-products (AGEs) of proteins that may result in protein dysfunction or activation of the receptor for AGE (RAGE) (2, 3). Lipid abnormalities also contribute to diabetes-associated atherosclerosis and even to insulin resistance (4). Dyslipidemia is associated with increased lipolysis and the release of higher amounts of non-esterified fatty acids (NEFA) into the bloodstream (5). Hyperglycemia creates a feedback loop increasing lipolysis (6; 7), leading to a chronic exposure to NEFA. Plasma NEFA promote a systemic insulin resistance state, susceptible to be modified by dietary or therapeutic intervention using fat-poor diets or hypolipidemic agents (8). Central obesity has been linked to predisposition to Type 2 diabetes, possibly through an increased lipolysis at visceral adipose tissue compared with subcutaneous adipocytes (9).

Despite the vast amount of evidence on the role that elevated serum levels of NEFA play on the development of vascular damage in diabetes (10), very little is known about their accumulation on the arterial wall. NEFA have been linked to changes in matrix proteoglycans leading to an increased lipoprotein uptake on the arterial wall (11). Emerging molecular imaging techniques, such as TOF-SIMS, rely on direct interfacing between thin tissue slices and a mass spectrometer as a detector, allowing precise measurements of previously unknown molecules (12; 13). Cluster TOF-SIMS has a strong bias towards hydrophobic molecules, displaying high-resolution images of the most abundant lipids present on the sample surface. Additionally, minimal manipulation of snap-frozen samples prevents analyte delocalization, critical for accurate co-localizations, allowing a straightforward integration with other histological techniques.

We have now used TOF-SIMS to characterize the presence and distribution of NEFA in atheroma plaque specimens form diabetic and non-diabetic subjects. The diabetic plaque samples had a more severe degree of inflammation and a higher amount of certain NEFA, including linoleic acid. NEFA co-localized with lipoprotein lipase (LPL) and MCP-1 expression in plaques and, in cultured VSMC, linoleic acid promoted NFκB activation and LPL and MCP-1 expression.

**RESEARCH DESIGN AND METHODS**

**Human subjects.** A total of 40 consecutive patients undergoing carotid endarterectomy (carotid stenosis >70%) at the Vascular Surgery Units of Hospital Clínico San Carlos (HCSC) and Fundación Jimenez Díaz (FJD) were studied. The study was approved by the local Ethical Committees in accordance with international guidelines and informed consent was obtained before enrolment. Basic patient characteristics are shown in table 1. There were no differences between patients with type 2 diabetes and non-diabetics in age, gender or prevalence of hypertension, hypercholesterolemia or smoking. Atherosclerotic plaques obtained during surgery were immediately processed for further studies. The first 4 diabetic and first 4 non-diabetic patient samples were snap-frozen immediately and named as test group and the next 32 collected were embedded in paraffin and named as
validation group. Both groups had similar clinical characteristics. Clinical data from the TOF-SIMS group are shown in table 2. Blood samples for biochemical analysis were drawn before the surgical procedure. NEFA were determined using the NEFA C enzymatic assay kit (WAKO GmbH, Neuss, Germany).

**TOF-SIMS**- Samples were kept at -80°C until 10 µm slices were cut using a cryostat (CM1900, Leica) at a constant temperature of -25°C. Tissue sections were deposited onto a stainless steel plate (15-7PH, Goodfellow, UK) and stored again at -80°C. After drying under a pressure of a few hPa during 15 minutes, they were directly analyzed in a TOF-SIMS V Mass Spectrometer (IonTof GmbH, Germany) fitted with a bismuth cluster ion source located at the Parque Cientifico de Barcelona.

The primary ions impinge the surface of the tissue section with a kinetic energy of 25 keV. The primary ion dose was between 4.7x10^{11} ions/cm² and 10^{12} ions/cm². The secondary ions were extracted with 2 keV energy and postaccelerated to 10 keV just before hitting the detector surface (single channel plate followed by a scintillator and a photomultiplier). A low-energy electron flood gun was activated to neutralize the surface during the analysis. The effective ion flight path is about 2 m using a reflectron and the mass resolution is about 6300 (FWHM) at m/z 35 and 10000 (FWHM) at m/z 795.7.

The field of view is 8000 x 8000 µm² (512x512 pixels). The name of the compounds or the m/z value of the peak centroid, the maximal number of counts in a pixel (mc) and the total number of counts (tc) are written below each image. The color scales correspond to the interval [0, mc].

Mass calibration and ion peak identifications were performed as has been described (13). Briefly, initial calibration of monatomic hydrogen was performed, followed by sequential calibration of known molecules up to 882 Da. In this paper we focus only in previously identified quasimolecular ions (14).

Total arterial wall NEFA was calculated as the mean of the different NEFA values normalized by the surface of the sample.

**Laser Capture Microdissection.** Frozen endartery tissue was sectioned at 10 µm and mounted on a slide covered with a polyethylene naphthalate membrane (PALMZeiss Microlaser Technologies, Germany). Tissue slices were stained with haematoxylin/eosin. RNase-free conditions were maintained as completely as possible.

TOF-SIMS-directed areas of interest were collected by laser microdissection and pressure catapulting (LMPC). LMPC was performed using a PALM MicroLaser system (PALM-Zeiss) containing a PALM MicroBeam (driven by PALM Micro-Beam software) and a PALM RoboStage. A typical setting used for laser cutting was a beam size of 30 m and laser strength of 30 mV under a 5x·ocular lens.

Dissected artery sections were catapulted directly into 25 µl of RNAqueous micro lysis solution, and total RNA was isolated according to the manufacturer’s recommendations (RNAqueous micro RNA isolation kit, Ambion, USA) and stored at -80 °C until use. Samples from 4 patients were studied in each group (diabetic vs. non-diabetic) with at least 3 areas of interest per patient, with a total surface of 120.000 µm².

**VSMC cultures.** Human aortic vascular smooth muscle cells (VSMC) (ATCC; CRL-1999) were cultured in Ham’s F-12 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, ITS (2.5 µg/mL insulin from bovine pancreas, 2.5 µg/L human transferrine, 2.5 ng/mL sodium selenite), 30 µg/mL endothelial growth supplement, at 37°C in 5% CO₂, as previously described (15). Cells between passages 3 and...
7 were used for all the experiments. Certain experiments were replicated in primary cultures of rat VSMC.

**RNA extraction and Real-Time Quantitative-Polymerase Chain Reaction.** Total RNA from VSMC cultures was isolated using TRizol Reagent (Invitrogen). One µg RNA was reverse transcribed with High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). RNA from tissue was isolated as described on the previous section. All Real-time PCR reactions were performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems) according to manufacturer's protocol using the DeltaDelta Ct method (16). Expression levels are given as ratios to 18S. Pre-developed primer and probe assays were obtained for human 18S and MCP-1 and LPL from Applied Biosystems.

**Immunohistochemistry.** Carotid atherosclerotic plaques were stored in paraformaldehyde for 24 hours and later in ethanol until paraffin-embedding. Immunohistochemistry was performed as previously described (17). Primary antibodies were mouse anti-LPL antibody (Abcam), rabbit anti-MCP-1 antibody (Immugenex), anti-smooth muscle cell a-actin (1A4; Dako), or anti-human macrophages (HAM-56; Dako). Negative controls using the corresponding IgG were included to check for nonspecific staining.

Computer-assisted morphometric analysis with the Olympus semiautomatic image analysis system Micro Image software (version 1.0 for Windows) was performed by a pathologist who was blinded to the patient’s group, as previously described (18). Results are expressed as percentage of positive staining per square millimeter.

**NF-κB Southwestern.** The distribution and DNA-binding activity of NF-κB in situ was detected using a digoxigenin-labeled double-stranded DNA probe with a specific NF-κB consensus sequence. Competition assays with 100-fold excess of unlabeled probe were used as negative controls. For colocalization studies, immunohistochemistry for macrophages was carried out on slides directly from the final wash of the Southwestern histochemistry protocol without allowing them to dry, as described (19).

**Electrophoretic Mobility Shift Assay (EMSA).** EMSA for NF-kB DNA binding activity was performed with nuclear protein extracts from VSMC as described (20). The specificity of the assay was tested with a 100-fold excess of unlabeled NF-kB consensus oligonucleotide added to the 32P-labeled probe-binding reaction.

**Western Blot and ELISA.** Equal amounts (30µg) of cell lysate protein were loaded onto 12.5% polyacrylamide gels, electrophoresed and transferred to nitocellulose membranes. Then, membranes were blocked with 7% milk powder in TBS-T for 1 hour and incubated with anti-LPL [Santa Cruz, Goat polyclonal sc-32382 used at 1:200], anti-IkBalpha [Santa Cruz Rabbit polyclonal sc-371 used at 1:1000] antibodies, overnight at 4°C. Membranes were washed with TBS-T and incubated with anti-goat, anti-mouse or anti-rabbit antibodies (1:2000) for 1 hour at RT. The signal was detected using a chemiluminiscence kit [GE Healthcare]. ELISA for MCP-1 (BD Biosciences) detection in culture supernatant was done accordingly with the manufacturer protocol.

**Statistical analysis.** Statistical analysis was performed with SPSS for Windows software package version 17 (SPSS Inc, Chicago, Ill). Results are expressed as the mean ± SD. In vitro experiments were performed at least three times. Statistical testing was done with a two-tailed α level of 0.05. Differences between diabetic and non-diabetic endarterectomy samples were assessed by the Mann-Whitney nonparametric test. Spearman correlation coefficients were calculated for continuous characteristics.
RESULTS

Increased NEFA in diabetic atherosclerotic plaques. Atheroma plaque samples subjected to TOF-SIMS Imaging analysis using bismuth clusters as primary ion source render multiple secondary ions corresponding with some of the most abundant metabolites present on their surface (Fig 1). Ionization using liquid ion guns is biased towards more hydrophobic metabolites rendering secondary ions. Therefore, we focused on surface analysis of lipids and lipid derivatives.

As shown on figure 1A, a density plot of the inorganic phosphate \((m/z 79)\) molecules can be created, similarly to any conventional histological study. The presence of phosphate ion correlates with the presence of biological tissue, and is comparable with the optical image in Figure 2. Thus, the morphometric measurement of inorganic phosphate can be used to estimate the size of the specimens. All metabolite measurements are expressed as ratio between metabolite area and phosphate area in order to normalize size variation. The phosphate area average value was \(1.835e^6 \pm 873000 \mu m^2\) in diabetes vs. \(2.54e^6 \pm 873000 \mu m^2\) in non-diabetics (p: ns).

Quantization of the relative abundance of molecules can be calculated, as their ionization efficiency is constant for the whole rastering. Significant differences between the molecules in diabetics and non-diabetics atheroma plaques were found in only 3 of the 16 molecules. These three molecules were NEFA: linoleic acid, palmitic acid and oleic acid.

The linoleate quasimolecular ion \((m/z)\) was consistently increased in all diabetic samples analyzed \((C18:2)\)(diabetics \(0.135 \pm 0.034\) vs. \(0.083 \pm 0.013\) non-diabetics, p<0.05). Also, palmitic acid \((C16:0)\) \((0.211 \pm 0.036\) vs. \(0.144 \pm 0.033; p<0.05)\) and oleic acid \((C18:1)\) \((0.184 \pm 0.078\) vs. \(0.106 \pm 0.017; p<0.05)\) were also significantly increased in plaques from the diabetics samples. As a control, synthetic triglycerides were irradiated at the ion dose used for analysis. Quantification of the in-source fragmentation rendered a negligible amount of NEFA (less than 10% of base peak). This effectively ruled out artifacts in NEFA measurement. The distribution pattern was similar between the 3 fatty acids, but did not fully overlap with triglyceride distribution.

Other lipids widely regarded as major players on atherosclerotic pathogenesis, such as cholesterol, did not significantly differ in quantity between diabetic and non-diabetic samples (Table 3). Furthermore, there were no differences in triglyceride accumulation (Table 3). Dyslipidemia in diabetes is mainly characterized by increased plasma triglyceride and NEFA levels (21; 22). To test whether NEFA plasma levels correlate with arterial wall NEFA, we measured enzymatically both protein-bound and protein-free plasma fatty acids. Total fasting plasma NEFA were higher in diabetics than in non-diabetics \((24.37 \pm 20.16\) vs \(14\pm 5.73\) mg/dL; p<0.05)(Table 1). We did not find any correlation between plasma NEFA and any of the lipid molecules measured by TOF-SIMS at the arterial wall (Suppl. table 1 which is available in the online appendix at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org)). The sum of NEFA present on the tissue correlate with glycemia but not with NEFA plasma levels (Suppl. table 1).

Lipoprotein lipase and MCP-1 expression on NEFA-rich areas. Several lipases promote the release of NEFA from complex lipids, mainly triglycerides. LPL is the major contributor to fatty acid hydrolysis in muscle and adipose tissue(23). High LPL expression by foam cells (24) plays a major role on local NEFA release in atherosclerotic vessels.

We used laser capture microdissection (LCM) in consecutive slices to those used for SIMS, to quantify mRNA expression in NEFA-rich areas identified by TOF-SIMS Imaging (Fig 2a). A consistent increase (2-
fold; p<0.05) in LPL mRNA expression was found in all diabetic NEFA-rich areas studied (Fig 2b) in comparison with non-diabetic NEFA-rich areas and control diabetic NEFA-poor areas.

The expression of MCP-1 provides an insight to plaque-associated inflammation. MCP-1 mRNA and protein were quantitated by LCM-qPCR (Fig 2d) and by immunohistochemistry (Fig 3a,c,e), respectively. MCP-1 mRNA expression was higher in diabetic NEFA-rich areas (Figure 2d). Diabetics had a significantly higher percentage of MCP-1 immunostained neointimal area when compared to non-diabetics (40±5 % vs 23±4 %, p<0.05).

Immunohistochemical analysis of LPL expression was carried out in paraffin-embedded sections; The area immunostained for LPL was markedly higher in diabetic than in non-diabetic patients (17.1±7.8 vs. 11.8±4.8% of neointimal area; P<0.05) (Fig. 3.b,d,f). Negative controls did not stain for LPL (not shown).

**Macrophage and T-cell infiltration in atherosclerotic lesions.** In relation to non-diabetic atherosclerotic samples, the diabetic group showed a significant increase of the percentage of neointima staining positive for macrophages (22 ± 3 % vs 15 ± 3 %, p<0.05) (Fig 4a & c), and T cells (4.4 ± 2 % vs 1.8 ± 0.5 %, p<0.05) (Fig 4b & d), which is consistent with the increased chemokine expression. Those results are similar to those found in the literature (25).

**NF-κB activation colocalizes with NEFA-rich areas.** NF-κB activation was localized to NEFA-rich areas by Southwestern in frozen samples consecutive to those use for TOF-SIMS allowing an overlay between them (Fig. 5.a,b). In addition, a higher number of nuclei staining positive for NF-κB activation with Southwestern histochemistry was noted in paraffin-embedded diabetes atherosclerotic plaques vs. non-diabetics (5140±512 vs. 3738±316 stained nuclei per mm², p< 0.05) (Fig.5.c-e). Nuclear staining was absent in negative controls (not shown).

**Linoleic acid activates NF-κB in VSMC and upregulates LPL and MCP-1 expression.** Additionally, we tested in cultured VSMC the possible functional relation between the observed colocalization of higher NEFA concentrations and NF-κB activation and LPL and MCP-1 upregulation. For these studies we chose linoleic acid, the NEFA with a highest increment in diabetic plaques (60% increase over non-diabetics).

Linoleic acid increased the NF-κB DNA-binding activity in nuclear extracts, as assessed by EMSA (2.2-fold at 90 µM LA, p<0.05) (Fig 6A,B). Confocal microscopy confirmed linoleic acid-induced NF-κB activation and nuclear translocation of p65-NF-κB (Fig 6C).

Linoleic acid increased LPL and MCP-1 mRNA and protein and this effect was prevented by pre-treatment with 2.5 µg/mL parthenolide, an NFκB inhibitor (28; 29) (Figure 7.A-D) (28; 29). This suggests that NFκB mediates linoleic acid actions on VSMC.

**DISCUSSION**

Our group reported recently the use of TOF-SIMS to study the cartography of lipids in atherosclerotic plaques (13). We now report the presence of NEFA-rich areas within human atherosclerotic plaques. These NEFA-rich areas are especially prominent in diabetics, and they colocalize with areas of inflammation characterized by NF-κB activation and increased LPL and MCP-1 expression.

The presence of NEFA-rich areas on the inner side of the endarterectomy specimens and its relation to inflammation is a novel finding, and very little in known about the mechanism involved. The extent of those NEFA-rich areas did not correlate with plasma NEFA levels, suggesting a local origin in the plaque. The distribution pattern of the
three main fatty acids was similar, but did not fully overlap with triglyceride distribution or VLDL deposition (unpublished observation). This is consistent with the hypothesis that, in addition to triglycerides, other complex lipids could act as NEFA precursors. However, we cannot exclude a triglyceride origin of NEFA. Several lipases such as LPL (30), endothelial lipase (31) and phospholipase A2 (32; 33) activate and release NEFA on atherosclerotic lesions and contribute to vascular injury. LPL is considered as the rate-limiting enzyme for hydrolysis of lipoprotein triglycerides (34), and it is likely to be secreted by macrophages within atherosclerotic lesions (24; 35). The increased expression of LPL in the NEFA-rich areas suggests that NEFA might be generated locally by the action of this enzyme. As lipid-laden macrophages become a major source of LPL (35). NEFA presence at the intima could be considered an indirect marker of macrophage infiltration. Nonetheless, image overlay shows a similar but not identical pattern for macrophages and NEFA, suggesting a contribution of other cell types, namely VSMC.

Diabetic patients had higher plaque levels of several NEFA probed (palmitic, linoleic and oleic acids), despite similar cholesterol and triglyceride levels to non-diabetics. In addition, diabetics had evidence for higher plaque inflammation (25). Furthermore, NF-κB activation and increased expression of MCP-1 mRNA were noted in NEFA-rich areas. This raises the question of the relationship between NEFA and inflammation that may be bi-directional. LPL may be expressed in the context of inflammation (23). In addition, linoleic acid activated NF-κB and increased the expression of LPL and MCP-1 in cultured VSMC. Recently, linoleate induction of cytokine expression by monocytes has been reported (36). The data are also consistent with the in vivo observation that an acute elevation of plasma NEFA activates the NF-κB pathway (37) and increases the hepatic expression of proinflammatory cytokines (38). NEFA may also contribute to vascular injury by inducing the synthesis of proteoglycan core proteins that promote LDL accumulation at the subendothelial layers (11). Very little is known about fatty acid signal transduction. In different experimental systems they have been postulated to bind to toll-like receptors particularly TLR4 (39; 40), PPARγ (41; 42) or fatty acid binding receptors (43) and to activate PKC θ (44; 45). These pathways converge on NF-kB activation and have been linked to inflammation and insulin resistance (30; 46).

In conclusion, diabetic atheroma plaques have a higher content in NEFA than those from non-diabetics. In vivo and in vitro experiments suggest that NEFA could be a key factor in the genesis of inflammation at the plaque.

ACKNOWLEDGMENTS

This work was supported by grants from Comunidad de Madrid (S2006/GEN-0247), European Network (HEALTH F2-2008-200647), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035; RD06/0014/0008) and Ministerio de Ciencia y Tecnología (SAF2007/63648), Eurosalud (EUS2005-0365), Fundación Mutua Madrileña and Fundación Ramón Areces. AO by Programa de Intensificación de la Actividad Investigadora (ISCIII/ Agencia “Laín Entralgo” Comunidad de Madrid)
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### TABLE 1. Baseline clinical characteristics of study participants.

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<thead>
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<th></th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>71±8</td>
<td>72±9</td>
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<tr>
<td>Sex (male/female)</td>
<td>16/3</td>
<td>18/3</td>
<td>ns</td>
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<tr>
<td>Hypertension</td>
<td>13/19</td>
<td>11/21</td>
<td>ns</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>14/19</td>
<td>12/21</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking</td>
<td>9/19</td>
<td>10/21</td>
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</table>

### TABLE 2. Baseline clinical characteristics of SIMS study participants. All values are expressed as mean±SD.

<table>
<thead>
<tr>
<th></th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>71±6.6</td>
<td>68.7±8.7</td>
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</tr>
<tr>
<td>Sex (male/female)</td>
<td>4/0</td>
<td>3/1</td>
<td>ns</td>
</tr>
<tr>
<td>Smoking</td>
<td>3/4</td>
<td>2/4</td>
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</tr>
<tr>
<td>Glycemia (mg/dL)</td>
<td>170.5±36.9</td>
<td>77.75±8.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Insulin (microIU/dL)</td>
<td>26±8</td>
<td>8.75±1.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>127.5±14.7</td>
<td>122.5±14.8</td>
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</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>63.7±9.6</td>
<td>65±3.5</td>
<td>ns</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>66.5±28</td>
<td>82±3</td>
<td>ns</td>
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<tr>
<td>LDL (mg/dL)</td>
<td>46.5±16.5</td>
<td>41±4.6</td>
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<td>Plasma Cholesterol (mg/dL)</td>
<td>132±42</td>
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<td>Plasma Triglycerides (mg/dL)</td>
<td>99.8±40</td>
<td>89±24.6</td>
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<tr>
<td>Apo A (mg/dL)</td>
<td>104.5±32</td>
<td>106±21</td>
<td>ns</td>
</tr>
<tr>
<td>Apo B (mg/dL)</td>
<td>58.5±15</td>
<td>65.6±9.2</td>
<td>ns</td>
</tr>
<tr>
<td>Plasma NEFA (mg/dL)</td>
<td>24 ± 20.16</td>
<td>14± 5.73</td>
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</tr>
<tr>
<td>Tissue NEFA (area NEFA/plaque size)</td>
<td>0.142±0.012</td>
<td>0.109±0.004</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Plaque size (µm²)</td>
<td>1835000±873500</td>
<td>2540000±873000</td>
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</table>
**TABLE 3.** Most abundant quasimolecular ions identified in atherosclerotic plaque negative spectrum (expressed as \(m/z\) values).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Negative spectrum ((m/z) values)</th>
<th>Signal in diabetics</th>
<th>Signal in non-diabetics</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoinositol fragment</td>
<td>223.0051</td>
<td>0.342 ± 0.069</td>
<td>0.277 ± 0.067</td>
<td>ns</td>
</tr>
<tr>
<td>Phosphoinositol fragment</td>
<td>241.0147</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0 (Palmitic acid)</td>
<td>255.2314</td>
<td>0.211 ± 0.036</td>
<td>0.144 ± 0.033</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>C16:1 (Palmitoleic acid)</td>
<td>253.2195</td>
<td>0.043 ± 0.005</td>
<td>0.033 ± 0.010</td>
<td>ns</td>
</tr>
<tr>
<td>C16:2 (Hexadecadioneic ac.)</td>
<td>251.2052</td>
<td>-</td>
<td>-</td>
<td>nq</td>
</tr>
<tr>
<td>C18:0 (Stearic acid)</td>
<td>283.2609</td>
<td>0.213 ± 0.015</td>
<td>0.181 ± 0.008</td>
<td>ns</td>
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<tr>
<td>C18:1 (Oleic acid)</td>
<td>281.2486</td>
<td>0.184 ± 0.078</td>
<td>0.106 ± 0.017</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>C18:2 (Linoleic acid)</td>
<td>279.2313</td>
<td>0.135 ± 0.034</td>
<td>0.083 ± 0.013</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>C20:4 (Arachidonic/Eicosatetraenoic)</td>
<td>303.2353</td>
<td>-</td>
<td>-</td>
<td>nq</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>385.3467</td>
<td>0.092 ± 0.028</td>
<td>0.061 ± 0.024</td>
<td>ns</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>429.3705</td>
<td>0.019 ± 0.008</td>
<td>0.012 ± 0.005</td>
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<td>-</td>
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<td>0.357 ± 0.020</td>
<td>0.319±0.021</td>
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<td>Phosphatidic acid</td>
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<td>Triglycerides</td>
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<td>0.380 ± 0.081</td>
<td>0.236 ± 0.050</td>
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<tr>
<td>Triglycerides</td>
<td>857.8163</td>
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*ns: non significant; nq: non quantifiable*
Figure 1. Representative secondary ion images obtained from carotid endarteries obtained from a non-diabetic (above) and a diabetic (below) patient, under the irradiation of bismuth cluster ions. From left to right: Tissue micrograph (A); Palmitate (C16:0) (B); Palmitoleate (C16:1) (C); Stearate (C18:0) (D); Oleate (C18:1) (E); Linoleate (C18:2) (F); Image overlay: Palmitoleate (Red), Cholesterol (Green), Triglycerides (Blue) (G); Phosphate (H); Phosphoinositol fragment (I); Triglycerides (J) and Total ion intensity (L). The field of view is 8 mm x 8 mm at 15,625 µm/pixel. See text for details.
Figure 2. Lipoprotein lipase and proinflammatory cytokine mRNA expression in NEFA-rich areas. (A&B) Hematoxilin-stained endarterectomy section for laser capture microdissection, showing NEFA-rich areas and its internal controls (24); LPL and MCP-1 mRNA levels (measured by quantitative reverse-transcription polymerase chain reaction) in NEFA-rich areas (n=6), diabetic VSMC control areas (n=6) and non-diabetic lipid-rich areas (n=6) microdissections (mean±95% confidence interval, p<0.05).
Figure 3. MCP-1 and LPL protein expression in diabetes. Representative examples of MCP-1 immunohistochemistry (IH) staining in a diabetic patient (A) and non-diabetic atherosclerotic patient (C) and LPL immunohistochemistry in diabetic (B) and non-diabetic (D). Magnification of a highly positive area within the lesion is shown for both stainings. The protein expression shows a significant increase on the diabetic group vs. the non-diabetic for MCP-1 (E) and LPL (F)(* p<0.05). Vessels form healthy controls did not stain positive (data not shown).
Figure 4. Inflammatory cells in carotid atherosclerotic plaques studied by immunohistochemistry. Consecutive sections were stained for macrophages (A&C) and T cells (B&D) on the plaque. A marked reduction in the number of positive cells within the intimae are observed on non-diabetics group vs. the diabetics group both for macrophages (C) and T-cell (D) (* p<0.05).
Figure 5. Arterial NF-κB activation determined by Southwestern histochemistry. Consecutive sections to those used in TOF-SIMS analysis (A upper right corner) were used for in situ southwestern (A), purple nucleus indicates activation, showing a similar pattern of distribution. The measured intensity is similar to the observed in paraffin (C&D). (E) Bar graph shows the quantification of positive nuclei per mm² in the lesions (D) (* p<0.05). P values refer to comparisons with non-diabetic patients. Negative control shows no staining.
Figure 6. NEFA induces NF-κB activation in VSMC. (A.) A representative EMSA is shown. (B) bar graph showing NF-κB activation in VSMCs incubated 3h with increasing Linoleate (LA) concentrations; (* p<0.05). (C) p65 subunit of NF-κB was detected by indireced immunostaining using FICT-labeled secondary antibodies and evaluated by confocal microscopy. Figure shows a representative experiment where rat VSMCs were incubated with/ without LA 90uM.
Figure 7. Linoleic acid (LA) upregulates MCP-1 and LPL expression at the mRNA and protein level in cultured VSMC. MCP-1 (A) and LPL (B) transcript induction after exposure to different concentrations (uM) of linoleic acid for 3 hours. C. Representative Western blot for LPL (above) and tubulin (below) as loading control D. Quantitation of LPL (Western blot, dark grey) and MCP-1 (ELISA; light grey) protein expression (* p<0.05). PTN: parthenolide.