When Lipid Metabolism Meets Inflammation: The Anti-inflammatory Power of Fat

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There is a large body of literature suggesting that monounsaturated fatty acids are beneficial to human diet, and may lower the risk of cardiovascular disease possibly through anti-inflammatory effects. From a biophysical point of view, unsaturated fatty acids are 'good' to biological membranes because, being liquid at body temperature yet not easily oxidized, help maintain membrane fluidity within the appropriate limits. Recent studies have revealed that, in animal models of metabolic disease, the adipose tissue releases the monounsaturated fatty acid palmitoleic acid (*cis*-9-hexadecenoic acid; 16:1*n-7*), which suppresses hepatic steatosis and improves insulin sensitivity in the whole body. However, aside from, or in addition to, these effects, there is something else to palmitoleic acid that makes it unique in terms of its biological activity, leading to the concept of this fatty acid serving as a lipid hormone, or 'lipokine' that coordinates metabolic responses between tissues.

4th Madrid Meeting on Dendritic Cells and Macrophages – National Center for Biotechnology, Madrid (Slide 1).

What you have in this slide (Slide 2) is a monocyte-derived human macrophage, stained in blue with a protein of lipid metabolism called lipin-1, which localizes on the surface of these huge cytoplasmic formations that tend to distribute in the periphery of the cells. These formations are lipid droplets and, as you can see, macrophages have many of them. If we take a closer look at one of these lipid droplets, what we see is something like this (Slide 3 – Lipid Droplets): a phospholipid monolayer decorated with a variety of proteins and inside a hydrophobic core composed of triglycerides (TAG) and cholesteryl esters (CE). Well, for many years these lipid droplets were thought of only as storage organelles for neutral lipids to be mobilized in the case of energy needs. Today we know that, in addition to that storage role, lipid droplets serve a wide variety of roles in cell physiology. For the purposes of this talk I will only highlight two of them. In the first place, lipid droplets may serve as signaling platforms for signaling enzymes to dock and interact; this is particularly true for lipid signaling enzymes; cytosolic phospholipase $A_2\alpha$, cyclooxygenase-2 or lipin-1, all localize to this organelle. In second place, lipid droplets have been found to play key roles in the development and progression of inflammatory metabolic disorders, of which the most common is cardiovascular disease (Slide 4 – Initiation of Atherosclerosis).

Atherosclerosis is a major cause for cardiovascular disease, and diabetes accelerates it. Atherosclerosis is initiated by the abnormal activation of endothelial cells, which is produced e.g. by increased lipid in the blood (dyslipidemia) or sugar in blood (diabetes). Endothelial cells release a wide variety of products with inflammatory potential that may attract monocytes and favor the interaction of these monocytes with the endothelial cells, which results in the infiltration of the activated monocytes into the vessel wall. There, the monocyte will differentiate into a macrophage and will take up enormous amounts of lipids that have been deposited into that space (primarily cholesterol esters), store them into lipid droplets thus becoming foam cells, and establishing an atheroma plaque. With time, smooth muscle cells from the tunica media will proliferate and reach the macrophage-rich area thus making things worse.

Among the many compounds secreted by endothelial cells that may affect monocytes there is one that interests

us in particular; actually it has been the focus of our interest for so many years now, and I bet that those in the audience who know me will have rapidly guessed what I am talking about: arachidonic acid (AA), of course. Endothelial cells secrete relative large amounts of this fatty acid (pathophysiological range 5-10 μM) (Slide 5 – Initiation of Atherosclerosis). So, what endothelial cell-derived AA does to circulating monocytes? We took our human monocytes and exposed them to 10 μM AA, as I just said, the pathophysiological concentration (Slide 6 – AA Induces Lipid Droplet Formation). Middle columns show the monocytes stained with DAPI to visualize their nuclei, and on the right column, you can see that monocytes exposed to this fatty acid produced lots of lipid droplets, stained in green with BODIPY. As a control, we also studied the effect of palmitic acid, a fatty acid that at much higher concentrations is proinflammatory. However at 10 μM it did not induce any lipid droplet formation, thus suggesting that the AA effect is somewhat specific. So these data provide an interesting concept, which is that the monocytes are bound to become a foam cell, and are starting to become one even before crossing the endothelial layer, and even before to becoming an actual macrophage. This adds an interesting twist to the diagram shown in the previous slide, I believe, because it indicates there may be foamy cells in circulation. If, using a sinple assay, we were able to recognize these foamy monocytes, i.e. by identifying some specific molecular signature/feature in them, we could count with an invaluable tool for early detection of cardiovascular disease (Slide 7 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). More on this in a moment…

Mass measurements confirmed that the AA-treated cells indeed produce elevated amounts of both TAG and CE (Slide 8 – AA Induces Neutral Lipid Formation). The fatty acid content of both TAG and CE was also analyzed by mass spec (Slide 9 – Fatty Acid Content of Triacylglycerol and Cholesteryl Esters). Fatty acids are expressed as number of carbons : number of unsaturations. You can see that, from a qualitatively point of view, the compositon profiles are very similar. The important thing in this slide is highlighted by the green box: 16:1, or palmitoleic acid. There is very little in resting cells, but it increases quite much in activated cells, hence we suspect it must bear some biological significance. For those in the audience who work in atherosclerosis, diabetes, obesity, or lipid metabolism in general, you all know that palmitoleic acid is one of the "rising stars" of the field (Slide 10 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). Palmitoleic acid has been suggested to function as a lipid hormone or adipokine, released by the adipose tissue to regulate lipid metabolism in liver and to improve insulin signaling. It has also been suggested to act to counteract inflammation, although this is a matter of debate. This fatty acid is getting so much recent interest that it is being marketing as "The New Good Fat", and can be found in Amazon.com and places like this… Well, in addition to all of this, our work adds to these results by showing that in response to an inflammatory challenge, circulating blood cells make palmitoleic acid and store it in significant quantities in the neutral lipids of lipid droplets (Slide 11 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). Who knows, maybe detection of elevated levels of 16:1 in circulating monocytes could provide us a with a reliable marker for foamy cell formation hence to identify individuals early at risk of cardiovascular disease.

(Slide 12 – Wait, there's more!). Yeah, more to the story. One day, the student I had in charge of this project decided he wanted to improve the method of separation and identification of palmitoleic acid by GC/MS. And by doing this, he found something as unexpected as remarkable, which is that cells do not have one palmitoleic acid; they actually have two… to be more precise, in addition to palmitoleic acid, cells make an unidentified second isomer. Importantly, depending on the lipid fraction under analysis, there were two peaks of 16:1 (phospholipids) or only one (neutral lipids) (Slide 13 – Two 16:1 Isomers in Macrophages). Comparison with commercial standards indicated that one was 16:1n-7 or palmitoleic acid proper and the other could either be 16:1n-10 (sapienic acid) or 16:1n-9 (Slide 14 – Two 16:1 Isomers in Macrophages). We made the DMOX derivative and analyzed it by electron impact MS. Confirming it was actually 16:1n-9 (Slide 15 – The Second Isomer is 16:1n-9). 16:1n-9 is a very unusual fatty acid, there is very little on it in PubMed.

What is the metabolic origin of 16:1n-9? (Slide 16 – Metabolic Origin of 16:1*n*-9). Since there are no Δ7 desaturases in mammalian cells, a likely possibility is that it derives from oleic acid (18:1*n*-9). The question was analyzed directly by using deuterated oleic acid (Slide 17 – Fatty Acid Methyl Ester (FAME) Fragmentation

Spectra). The spectra from oleic acid and its deuterated variety are qualitatively the same, it's only that the fragments of the deuterated fatty acid obviously appear at higher m/z ratios. Incubation of the cells resulted in the appearance of a second deuterated fatty acid with the expected mass of 16:1n-9 and characteristic fragments consistent with it being 16:1n-9 (Slide 18 – Fatty Acid Methyl Ester (FAME) Fragmentation Spectra). Finally, if oleic acid is the actual precursor of 16:1n-9 this has to coccur via β-oxidation; etomoxir, an inhibitor of this route, blunts 16:1n-9 accumulation (Slide 19 – Effect of Etomoxir on 16:1n-9 Accumulation). So, what these data are teling us? It seems that strikingly, during activation, immunoinflammatory stimuli activate both biosynthesis and discrete metabolism of fatty acids to generate diversity (Slide 20 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism).

As indicated before, there is a fundamental difference in the distribution of these two isomers among lipid classes, the n-9 isomer localizes in both phospholipid and neutral lipids, whereas n-7 only appears in phospholipid (Slide 21 – Distribution of 16:1 Isomers Between Lipid Classes). This distribution of n-9 is quite peculiar since no other fatty acid distributes similarly (Slide 21 – Distribution of 16:1 Isomers Between Lipid Classes). When we analyzed the distribution of n-9 in zymosan-treated cells, we found that it increases in all lipid classes but the largest increases are in neutral lipids (Slide 22 – Distribution of 16:1 Isomers Between Lipid Classes).

The unique distribution of 16:1n-9 among cellular lipids and the finding that its levels are increased during cellular activation suggest a specific biological role for this unusual fatty acid. To study this question, we prepared cells enriched in this fatty acid by incubating them with 10 μM 16:1n-9 for 2 h in serum-free medium. This procedure results in the cells taking up the fatty acid and preferentially accumulating it in neutral lipids, in a similar manner as if they had been previously activated with a receptor-directed stimulus (Slide 23 – Assessing the Biological Effects of 16:1n-9). Then, the cells were stimulated with LPS and the effects on the expression of a number of proinflammatory genes was investigated (Slide 24 – 16:1n-9 Possesses Anti-inflammatory Properties *in vitro*). Cells enriched in 16:1n-9 showed significant decreases in the expression of all genes tested, and such decreases were generally comparable to those found in the 22:6n-3-treated cells. 16:1. 16:1n-9 was significantly more potent than 16:1n-7 for all genes tested; 16:1n-7 had significant effects only in two of them, Tnf and Nos2. These data show that 16:1n-9 has a spectrum of biological activity that is clearly distinguishable from that of 16:1n-7. We also conducted experiments with mice (Slide 25 – 16:1n-9 Possesses Anti-inflammatory Properties *in vivo*). In these experiments, the fatty acid was administered i.p. to mice 1 h before i.p. injection of LPS for 6 h. Afterward, the animals were sacrificed, peritoneal cells were harvested, cell samples matched by protein content, and the expression levels of Il6 were studied. Both 16:1n-9 and 22:6n-3 inhibited Il6 gene expression by the peritoneal cells isolated after the LPS challenge. Analysis of serum IL-6 protein confirmed a strong decrease in the amount of circulating IL-6 protein in the 16:1n-9-treated mice. Unexpectedly, IL-6 protein levels in serum from 22:6n-3 treated cells were no different from those in serum from control untreated animals.

So, in conclusion, these results suggest that the relatively uncommon fatty acid acid 16:1n-9 may possess anti-inflammatory activity that could be comparable to that of omega-3 fatty acids, and clearly distinguishable from that of palmitoleic acid. From a biochemical point of view this fatty acid shows unique characteristics, such as preferentially accumulating in neutral lipids and being produced 'on demand' via unique roues involving both elongation and β-oxidation (Slide 26 – 16:1n-9 as a novel anti-inflammatory fatty acid). Just to conclude, the Acknowledgments slide (Slide 27 – Acknowledgments). Thank you very much for your attention.

REFERENCES

1. Valdearcos, M., E. Esquinas, C. Meana, L. Gil-de-Gómez, C. Guijas, J. Balsinde, and M. A. Balboa. 2011. Subcellular localization and cellular role of lipin-1 in human macrophages. *J. Immunol*. 186: 6004-6013.

2. Valdearcos, M., E. Esquinas, C. Meana, L. Peña, L. Gil-de-Gómez, J. Balsinde., and M. A. Balboa. 2012. Lipin-2 reduces proinflammatory signaling induced by saturated fatty acids in macrophages. *J. Biol. Chem.* 287: 10894–10904.
3. Meana, C., L. Peña, G. Lordén, E. Esquinas, C. Guijas, M. Valdearcos, J. Balsinde., and M. A. Balboa. 2014. Lipin-1 integrates lipid synthesis with proinflammatory responses during TLR activation in macrophages. *J. Immunol.* 193: 4614–4622.
4. Guijas, C., G. Pérez-Chacón, A. M. Astudillo, J. M. Rubio, L. Gil-de-Gómez, M. A. Balboa, and J. Balsinde. 2012. Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A_2-dependent synthesis of lipid droplets in human monocytes. *J. Lipid Res.* 53: 2343–2354.
5. Guijas, C., J. P. Rodríguez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2014. Phospholipase A_2 regulation of lipid droplet formation. *Biochim. Biophys. Acta* 1841: 1661–1671.
6. Pérez-Chacón, G., A. M. Astudillo, D. Balgoma, M. A. Balboa, and J. Balsinde. 2009. Control of free arachidonic acid levels by phospholipases A_2 and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 1791: 1103-1113.
7. Astudillo, A. M., D. Balgoma, M. A. Balboa, and J. Balsinde. 2012. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim Biophys. Acta* 1821: 249-256.
8. Balsinde, J., and M.A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells. *Cell. Signal*. 17: 1052-1062.
9. Balsinde, J., and E. A. Dennis. 1996. The incorporation of arachidonic acid into triacylglycerol in $P388D_1$ macrophage-like cells. *Eur. J. Biochem*. 235: 480-485.
10. Balsinde, J., B. Fernández, and J.A. Solís-Herruzo. 1994. Increased incorporation of arachidonic acid into phospholipids in zymosan-stimulated mouse peritoneal macrophages. *Eur. J. Biochem.* 221: 1013–1018.
11. Astudillo, A. M., G. Perez-Chacón, D. Balgoma, L. Gil-de-Gómez, V. Ruipérez, C. Guijas, M. A. Balboa, and J. Balsinde. 2011. Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells. *Biochim. Biophys. Acta* 1811: 97-103.
12. Astudillo, A. M., G. Pérez-Chacón, C. Meana, D. Balgoma, A. Pol, M. A. del Pozo, M. A. Balboa, and J. Balsinde. 2011. Altered arachidonate distribution in macrophages from caveolin-1 null mice leading to reduced eicosanoid synthesis. *J. Biol. Chem.* 286: 35299–35307.
13. Gil-de-Gómez, L., A. M. Astudillo, C. Meana, J. M. Rubio, C. Guijas, M. A. Balboa, and J. Balsinde. 2013. A phosphatidylinositol species acutely generated by activated macrophages regulates innate immune responses. *J. Immunol*. 190: 5169–5177.
14. Gil-de-Gómez, L., A. M. Astudillo, C. Guijas, V. Magrioti, G. Kokotos, M. A. Balboa, and J. Balsinde. 2014. Cytosolic group IVA and calcium-independent group VIA phospholipase A_2s act on distinct phospholipid pools in zymosan-stimulated mouse peritoneal macrophages. *J. Immunol*. 192: 752–762.
15. Pérez-Chacón, G., A. M. Astudillo, V. Ruipérez, M. A. Balboa, and J. Balsinde. 2010. Signaling role for lysophosphatidylcholine acyltransferase 3 in receptor-regulated arachidonic acid reacylation reactions in human monocytes. *J. Immunol*. 184: 1071-1078.
16. Balboa, M. A., R. Pérez, and J. Balsinde. 2008. Calcium-independent phospholipase A_2 mediates proliferation of human promonocytic U937 cells. *FEBS J*. 275: 1915–1924.
17. Balgoma, D., A. M. Astudillo, G. Pérez-Chacón, O. Montero, M. A. Balboa, and J. Balsinde. 2010. Markers of monocyte activation revealed by lipidomic profiling of arachidonic acid-containing phospholipids. *J. Immunol.* 184: 3857-3865.
18. Balgoma, D., O. Montero, M. A. Balboa, and J. Balsinde. 2008. Calcium-independent phospholipase A_2-mediated formation of 1,2-diarachidonoylglycerophosphoinositol in monocytes. *FEBS J*. 275: 6180–6191.
19. Balgoma, D., O. Montero, M. A. Balboa, and J. Balsinde. 2010. Lipidomic approaches to the study of phospholipase A_2-regulated phospholipid fatty acid incorporation and remodeling. *Biochimie* 92: 645–650.

20. Casas, J., C. Meana, E. Esquinas, M. Valdearcos, J. Pindado, J. Balsinde, and M. A. Balboa. 2009. Requirement of JNK-mediated phosphorylation for translocation of group IVA phospholipase A_2 to phagosomes in human macrophages. *J. Immunol.* 183: 2767–2774.
21. Casas, J., M. Valdearcos, J. Pindado, J. Balsinde, and M. A. Balboa. 2010. The cationic cluster of group IVA phospholipase A_2 (Lys488/Lys541/Lys543/Lys544) is involved in translocation of the enzyme to phagosomes in human macrophages. *J. Lipid Res*. 51: 388–399.
22. Rubio, J. M., J. P. Rodríguez, L. Gil-de-Gómez, C. Guijas, M. A. Balboa, and J. Balsinde. 2015. Group V secreted phospholipase A_2 is up-regulated by interleukin-4 in human macrophages and mediates phagocytosis via hydrolysis of ethanolamine phospholipids. *J. Immunol.* 194: 3327–3339.
23. Guijas, C., A. M. Astudillo, L. Gil-de-Gómez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2012. Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages: comparison with arachidonic acid. *Biochim. Biophys. Acta* 1821: 1386–1393.
24. Guijas, C., C. Meana, A. M. Astudillo, M. A. Balboa, and J. Balsinde. 2016. Foamy monocytes are enriched in cis-7-hexadecenoic fatty acid (16:1n-9), a possible biomarker for early detection of cardiovascular disease. *Cell Chem. Biol*. 23: 689–699.

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