Anti-inflammatory Lipids in Action: Palmitoleic Acid in Immune Modulation

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Inflammation underlies the progression of many chronic disorders with macrophages serving as key effectors. Palmitoleic acid (16:1n-7) has been linked to anti-inflammatory activity, though the molecular basis remains unclear. In phagocytic cells, most of 16:1 n-7 is incorporated into a distinct phosphatidylcholine species, PC(16:0/16:1 n-7), suggesting that its function may depends on this lipid. Synthetic phospholipid experiments reveal that this PC species directly modulates macrophage activation, dampening NF-κB signaling, reshaping transcriptional programs, and promoting an M2-like, anti-inflammatory phenotype with enhanced phagocytosis. These actions persist in ether analogs resistant to phospholipase cleavage, suggesting that free 16:1 n-7 release is unnecessary. Together, these findings uncover a lipid-based mechanism of immune regulation, where the structural features of PC(16:0/16:1 n-7) confer intrinsic activity. This work expands the concept of membrane phospholipids as active regulators of immunometabolism and highlights the therapeutic promise of defined lipid species for macrophage reprogramming in inflammatory disease.

Transcription of the lecture presented on Thursday, December 11, 2025 at the Symposium Course *"Resolution of Inflammation in Cardiovascular and Multiorgan Pathology", held in Madrid, Spain* (**Slide 1**).

Since this symosium is organized by members of CIBERCV, I found it very appropriate to start my talk with a slide depicting the initiation of atherosclerosis (**Slide 2** – Initiation of Atherosclerosis). In this scenario, endothelial cells behave abnormally, which is due to e.g. 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 from oxidized LDLs), 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 arachidonic acid (AA) (**Slide 3** – Initiation of Atherosclerosis). Damaged endothelium secretes relatively large amounts of this fatty acid, with the capacity to activate monocytes. So, what does this AA do to the monocytes? We showed a few years ago that AA promotes a foamy cell phenotype to the monocytes. 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 (**Slide 4** – Circulating Foamy Cells). Thus it came to us that if, using a simple assay, we were able to recognize these foamy monocytes, i.e. by identifying some specific molecular signature/feature/marker in them, we could count with an invaluable tool for early detection of cardiovascular disease. And, since we are lipidologists, we decided to analyze the lipidome of these foamy monocytes.

And here is when our story with palmitoleic acid started. In this slide you can see the expression of all four genes involved in fatty acid synthesis, namely ACC, FAS, ELOVL5 and SCD-1 is increased in AA-treated monocytes (**Slide 5** – Arachidonic Acid Up-regulates the Expression of Lipogenic Genes). This brings about an increase in

the cellular synthesis of fatty acids, which in turn will be packed into neutral lipids and stored as cytoplasmic lipid droplets (**Slide 6** – Arachidonic Acid Induces Lipid Droplet Formation). Middle columns show the monocytes stained with DAPI to visualize their nuclei, and on the right column, you can see the 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.

Mass measurements confirmed that the AA-treated cells indeed produce elevated amounts of both TAG and CE (**Slide 7** – Arachidonic Acid Induces Neutral Lipid Formation). We can go a bit further, and analyze the molecular composition of these fractions, which is to say that we can analyze their fatty acid profile (**Slide 8** – Fatty Acid Content of Triacylglycerol and Cholesterol Esters). Fatty acids are expressed as number of carbons : number of unsaturations. You can see that, from a qualitatively point of view, the fatty acid profiles in both TAG and CE 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.

So, if palmitoleic acid accumulates to this extent in neutral lipids, it should mean that there is lots of this fatty acid in the membranes of the cells, as membranes are supposed to be the primary targets for fatty acid incorporation (at least in phaggocytes), lipid droplets being sort of storage depots for excess fatty acids. And, on the other hand it was interesting that it had to be precisely palmitoleic acid, because at the time we found this, palmitoleic acid was definitely a "rising star" in the field (**Slide 9** – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). Palmitoleic acid was defined 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 controversy because some of the data with animals do not correspond to data from humans, the things is that if you go to Amazon.com or similar on-line stores, you can purchase a big can of omega-7, as much as you want. I do not know if you can see it, but here in the label it says: "The New Good Fat", which is a very nice catch phrase, I think… 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 10** – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). But there is much more to this, as I am going to show you in a moment.

One day, one of my students decided he wanted to improve the chromatographic method for separation of palmitoleic acid. And you see here that he succeeded and there is much separation between the 16:1 peak and others in this neutral lipid sample. (**Slide 11** – Two 16:1 Isomers in Monocytes?). The surprise came when he analyzed next a membrane fraction, that is, phospholipids –where actually most of the fatty acids should be, at least those that matter in signaling–, and saw that there was not one palmitoleic acid; there were two palmitoleic acid peaks. That means that, in addition to palmitoleic acid, the cells make an unidentified second isomer. Comparison with commercial standards indicated that one was actually 16:1n-7 or palmitoleic acid proper and the other could either be 16:1n-10 (sapienic acid) or 16:1n-9 (hypogeic acid). To distinguish between these two possibilities we made dimethyl disulfide derivatives of the fatty acid methyl esters and analyzed them by gas chromatography coupled to electron impact mass spectrometry (**Slide 12** – Analysis of 16:1 Isomers. Dimethyl Disulfide Adducts). The good thing about this derivatization is that sulfide groups add to the double bound preventing possible isomerizations, and fragmentation occurs precisely in between the methyl sulfides, i.e. where the double bond was. This generates specific fragments for each isomer which allows us to specifically identify the isomer in question: the omega end fragment, the carboxy-end fragment, and a third one that derives from the carboxy end by loss of methanol. And, another great thing, the disulfide derivatives resolve very well from each other in gas chromatography. Application of this technique to analyze the occurrence of 16:1 isomers in human monocytes indicated that actually, the monocytes contained high levels of n-7 and n-9 and just a blip of n-10 (**Slide 12** – Analysis of 16:1 Isomers. Dimethyl Disulfide Adducts). Given its low levels, I will ignore sapienic acid for the purposes of this talk. Here is the distribution of the 16:1 fatty acid among lipid fractions. In monocytes

(**Slide 13** – Distribution of 16:1 Isomers in Human Monocytes). You see 16:1n-9 but not 16:1n-7 in neutral lipids, but the bulk of both fatty acids is in the membrane phospholipids, as presumed, which is where one would expect them to be if they play any signaling role (which is what we are interested in).

At this point, the burning question here is, what is the relevance of these fatty acids? (**Slide 14** – What Is the Biological Relevance). Basically, we started by doing something quite simple: we enriched the cells with these fatty acids and looked at gene expression (**Slide 15** – Assessing the Biological Effects of 16:1 Fatty Acids). The cells were stimulated with LPS and the effects on the expression of a number of proinflammatory genes was investigated (**Slide 16** – 16:1 Fatty Acids Possess Anti-inflammatory Properties *in vitro*). As a control for these experiments we also used cells enriched in DHA (22:6n-3), an omega-3 fatty acid that is well established to possess anti-inflammatory activity. Cells enriched in 16:1n-9 fatty acids 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. 16:1n-9 was significantly more potent than 16:1n-7 for all genes tested. We also conducted experiments with mice (**Slide 17** – 16:1 Fatty Acids 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.

For better or worse, we are lipid biochemists, and a key question we wanted to address is how cells handle their 16:1 content. Do they use specific molecular species to store it? Do they mobilize it upon activation? Do they remodel it among phospholipids? You know, all those things that fascinate lipidologists (**Slide 18** – Cellular Utilization of 16:1 Fatty Acids by Phagocytic Cells). As we already know that the bulk of 16:1 is in phospholipids, we conducted a full phospholipidomic analysis of the cells by mass spectrometry, both GC-MS and LC-MS (**Slide 19** – Distribution of 16:1 Fatty Acids Among Phospholipids). And the first thing that caught our attention is that the vast majority of 16:1 fatty acids are localized in PC phospholipids; minor amounts in PE, PI or PS, and lots in PC. Not only that, when we analyzed species by LC-MS, we found that ~75% of total cellular 16:1 is contained within one single species, PC(16:0/16:1). This is unusual and seems to suggest that such a comprtmentalization may have some biological meaning. The next thing we did was to analyze the levels of 16:1 among species after stimulating the cells with opsonized zymosan, a classic phagocytic stimulus. What we saw is that the levels of 16:1 in PC(16:0/16:1) significantly decreased while significantly increasing in two PI species (**Slide 20** – Changes in 16:1-containing Species in Human Monocytes). So, when the cells become activated there is a net transfer of 16:1 from PC to PI. Please believe me, for a lipidologist like myself, this is a fascinating finding. So we proceeded to characterize the mechanism involved. To make a long story short I will just jump straight to the end and present the biochemical pathway we worked out to explain this phenomenon (**Slide 21** – Mechanism of 16:1 Trafficking from PC to PI). In the first place, a phospholipase A_2, iPLA$_2$β, releases 16:1 from PC(16:0/16:1), and the free 16:1 is converted into fatty acyl-CoA and incorporated by an acyltransferase using the abundant lysoPI acceptors being produced during activation by cPLA$_2$α, another different PLA$_2$ form. This pathway is quite significant for two reasons. First, it constitutes one of not many examples for two signal-regulated PLA$_2$s doing different things, but acting in concert within the same biochemical pathway to generate lipid diversity, and (ii) something I am sure it will interest some of you quite much, that the major enzyme liberates anti-inflammatory 16:1 is precisely also the major effector for DHA release, the precursor of many SPMs (**Slide 22** – PLA$_2$-Regulated Lipid Signaling Pathways). So, here we have cPLA$_2$α in charge or regulating eicosanoid production, mostly pro-inflammatory, and this side the iPLA$_2$β, regulating the release of anti-inflammatory fatty acids, 16:1 and DHA.

Well, I said before that the the finding that one single phospholipid species comprises as much as 75% of total content of a fatty acid in a cell was unusual. Yes it is, and it got us thinking what would happen to the anti-inflammatory ability of the cells if, instead of adding the free fatty acid, we added that phospholipid instead, the

whole phospholipid. And this makes sense, because if we were to think in the posible therapeutic potential of 16:1 gatty acids, delivery of a phospholipid is preferable over that of a fatty acid because od toxicity issues. We synthesized in the lab the required lipid, both of them, and added them to the macrophages (**Slide 23** – Chemical structures of 16:1-containing Phospholipids). I have to say, from now on all the data I am going to present was carried out in mouse peritoneal macrophages, not human monocytes, as I had shown up to this point. We worked out the conditions for the incorporation of the phospholipids to the macrophages, and we got conditions that results in the cells taking them up quite well, resulting in the specific increase of the constituent fatty acids, 16:0 and 16:1, and no other, implying that they are taken up and stay, they are not metabolized to anything else (**Slide 24** – Incorporation of PC(16:0/16:1n-7). Also, 16:1n-7 increases only in phospholipids and in the PC fraction (**Slide 25** – Incorporation of PC(16:0/16:1n-7). In summary, the cells take up the phospholipid and retain it in its original form; metabolism is negligible.

We exposed the cells to LPS and measured IL-6 and TNF-α expression both at gene and protein expression levels; you can there is a strong inhibitory effect. Note that we also used phospholipids that contained oleic acid or palmitic acid as controls, and neither of these exerted any significant effect (**Slide 26** – Anti-inflammatory activity of AA-containing PC). These data thus confirmed the specific anti-inflammatory action of the 16:1 fatty acids. And the effect is not stimulus-speficic; it occurs regardless of the stimulant employed, whether receptor-directed or soluble (**Slide 26** – Anti-inflammatory activity of AA-containing PC). So, the phospholipid works pretty much the same as the free fatty acid.

We said a while ago that 16:1 jumps from PC to PI during activation. So the question is obvious: is this 16:1n−7 remodeling pathway from PC to PI is relevant for the fatty acid to exert its anti-inflammatory effects, experiments were conducted in which the PC(16:0/16:1n−7)-loaded cells were exposed to LPS in the presence of two structurally unrelated iPLA$_2$β inhibitors, to prevent the movement of 16:1n−7 among phospholipid classes. We also used an inhibitor of Acyl-CoA synthetase. Neither of the inhibitors affected the anti-inflammatory effect of the phospholipid (**Slide 27** – What Is the Bioactive Form of Palmitoleic Acid?). These data suggested that PC(16:0/16:1) hydrolysis by iPLA$_2$β is not required for an anti-inflammatory effect. To substantiate this suggestion, we prepared cells deficient in iPLA$_2$β by using antisense inhibition, which is a well established approach. You see again that, when challenged with LPS, both iPLA$_2$β-deficient and normal cells exhibited similar inhibitory responses to PC(16:0/16:1n−7), underscoring the lack of iPLA$_2$β involvement in this effect. To obtain further evidence for this, we went on to synthesize a PC molecule in which the 16:1n−7 lateral chain is linked to the glycerol backbone not by a classical ester bond but by an ether bond, PC(16:0/O-16:1n−7) (**Slide 27** – What Is the Bioactive Form of Palmitoleic Acid?). This modification makes the phospholipid resistant to phospholipase attack and, hence, unable to release the 16:1n−7 moiety. And… yo can see here that, when used under identical conditions, both PC(16:0/16:1n−7) and the ether analog, PC(16:0/O-16:1n−7), inhibited the LPS-induced gene expression to comparable levels. These results show that the bioactive form of 16:1n−7 arises from its incorporation into a phospholipid rather than from the formation of a free fatty acid.

Nuclear factor kappa B (NF-κB) plays a central role in regulating the transcription of proinflammatory genes in macrophages. When activated, the p65/p50 heterodimer of NF-κB translocates to the nucleus, where it drives gene expression. So we measured p65 translocationin macrophages by immunofuorescence. Blue is DAPI which marks the nucleus, p65 staining is in red, and co-localization is white. I know the figure is hard to see, but we have the quantification on the right hand side that can help a bit (**Slide 28** – 16:1-containing PC Inhibits NFκB translocation). Immunofluorescence staining of p65 in unstimulated macrophages showed largely a cytoplasmic location; you see there is no white spots at all. However when treated with LPS you see the white spots, indicating that p65 translocated to the nucleus, and these white spots are greatly reduced in the PC(16:0/16:1n-7)-enriched cells. Here is the exact same experiment, using PC(16:0/16:1n-9) instead of PC(16:0/16:1n-7): the exact same result (**Slide 29** – 16:1-containing PC Inhibits NFκB translocation). Please note that even in cells loaded with the phospholipid but otherwise left untreated (i.e., not exposed to LPS), the extent of nuclear location of p65 was lower than in control cells. This suggests that simply enriching the cells with PC(16:0/16:1n–7), or

PC(16:0/16:1n–9), is enough to reduce basal NF-κB activity. Given that NF-κB is a hallmark of proinflammatory gene expression, we speculated that reduced activity of this transcription factor would conceivably skew the cells to a more pronounced anti-inflammatory character. Thus we decided to analyze the expression of two widely used markers for alternatively activated macrophages Ym1 (Chi3l3) and Fizz1 (Retnla) [46] by qPCR. Both markers were found to be markedly up-regulated in the PC(16:0/16:1n–7)-loaded macrophages compared to untreated cells (**Slide 30** – 16:1-containing PC Promotes an Anti-inflammatory Profile). In contrast, Il10, another anti-inflammatory gene, was unaffected, however, that the LPS-induced up-regulation of this gene was further enhanced in the PC(16:0/16:1n–7)- or PC(16:0/16:1n–9)-loaded cells (**Slide 30** – 16:1-containing PC Promotes an Anti-inflammatory Profile). Together, these results suggest that loading the macrophages with 16:1-containing PC intensifies an anti-inflammatory character. To further characterize the PC(16:0/16:1n–7) effects, we conducted transcriptional mRNA profiling in murine macrophages under conditions that lead to an anti-inflammatory M2-like phenotype (IL-13 plus IL-4, 20 ng/ml, each for 12 h). To assess gene expression changes associated with M2 polarization, we analyzed a number of genes widely characterized to increase during M2 polarization by RNAseq in cells incubated with or without phospholipid (**Slide 30** – 16:1-containing PC Promotes an Anti-inflammatory Profile). The findings clearly demonstrated that both PC(16:0/16:1n–7) and PC(16:0/16:1n–9) substantially enhance the anti-inflammatory properties of macrophages by modulating gene expression patterns associated with alternative polarization. One of the genes in this list, *Mrc1*, codes for CD206 a mannose receptor. Flow cytometry analyses of the expression of this receptor confirmed it is increased in the 16:1-containing PC-treated cells (**Slide 30** – 16:1-containing PC Promotes an Anti-inflammatory Profile).

Enhanced phagocytosis is a hallmark of alternatively activated (M2) macrophages, setting them apart from their pro-inflammatory counterparts. Therefore, we aimed to assess next whether loading macrophages with 16:1-containing PC would induce a heightened phagocytic response. The 16:1-containing PC-loaded macrophages were exposed to fluorescent yeast-derived zymosan as a phagocytic challenge, and analyzed by confocal microscopy (**Slide 31** – 16:1-containing PC Enhances Zymosan Phagocytosis). Cells loaded with both kinds of phospholipid species manifested a significantly enhanced phagocytic response. Parallel experiments utilizing cells loaded with either PC(16:0/18:1n–9) or PC(16:0/16:0) showed no enhanced phagocytic activity compared to otherwise untreated control cells, thus highlighting the specificity of action of the 16:1 moiety.

So in the end, this slide summarizes all I have been telling you: palmitoleic acid and its positional isomer hypogeic being bioactive as long as the are part PC(16:0/16:1), they promote the macrophage towards an anti-inflammatory, potentially pro-resolutory state which underscores the pharmacotherapeutic potential of defined lipid species in reprogramming macrophage function in inflammatory diseases (**Slide 32** – Anti-inflammatory Fatty Acids 16:1n-7, 16:1n-9).

To conclude, thanks to the palmitoleic crew of my lab… (**Slide 33** – Acknowledgments). Thanks as well to our collaborators and our sponsors (**Slide 34** – Acknowledgments)... As requested, a comprehensive list of significant papers from our laboratory follows.

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