An Unexpected Variety of Palmitoleic Acid Isomers in Human Inflammatory Cells.

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Phagocytic cells contain an unexpected variety of 16:1 isomers, which can be distinguished on the basis of their biological activity and cellular regulation. While it is clear that much work needs to be carried out to unravel the effects of 16:1 fatty acids on innate immune reactions, the elucidation of intracellular metabolic routes utilizing these fatty acids might provide interesting opportunities of intervention to ameliorate the inflammatory response.

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(**Slide 1**).

This slide (**Slide 2** – Human Monocyte-Derived Macrophage) shows 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 cholesterol 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 arachidonic acid (AA), of course. Damaged endothelium secretes relatively large amounts of this fatty acid, with the capacity to activate monocytes. So, what does micromolar AA do to the monocytes? Well, many things of course, but a very important one for the purposes of this tal is that AA increases the expression of all four genes involved in fatty acid synthesis, namely ACC, FAS, ELOVL and SCD (**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. 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 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 (**Slide 7** – Circulating Foamy Cells). 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.

Mass measurements confirmed that the AA-treated cells indeed produce elevated amounts of both TAG and CE (**Slide 8** – 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 9** – 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.

And it is interesting that it had to be precisely palmitoleic acid, because 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 controversy because some of the data with animals do not correspond to data from humans, the thing 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, true or not, seems like 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 11** – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism). Who knows, maybe detection of elevated levels of 16:1 in circulating monocytes by doing lipidomic analyses in blood samples could provide us with that reliable marker for foamy cell formation hence to identify individuals early at risk of cardiovascular disease (**Slide 12** – Lipid Profile Test), and currently we are doing things in this direction to assess how robust this possible marker could be.

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 13** – Two 16:1 Isomers in Monocytes?). The surprise came when he analyzed next a membrane fraction, that is, phospholipids, 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. 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 14** – 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 frgamentation 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 (**Slide 15** – Analysis of 16:1 Isomers. Dimethyl Disulfide Adducts).

Application of this technique to analyze the occurrence of 16:1 isomers in human monocytes indicated that actually, the monocytes contained the three isomers: high levels of n-7 and n-9 and much lower levels of n-10 (**Slide 16** – Analysis of 16:1 Isomers. Human Monocytes). Because of this difference, we focused on the n-9 isomer, although I am not forgetting the n-10 isomer, and will come back to it later in my talk. Let me tell you, the identification of relatively high levels of the omega-9 in monocytes was quite a surprise, since 16:1n-9 is a very unusual fatty acid in the animal kingdom, it is almost nowhere to be found. In addition, the identification of this fatty acid created some sort of metabolic problem (**Slide 17** – Metabolic Origin of 16:1n-9. No Δ7 Desaturases in Mammals). We do not have the enzyme that makes this isomer directly from palmitic acid. We have Δ^9 isomerases that make palmitoleic, and we have Δ^6 desaturases that make the omega-10 isomer sapienic acid, but we do not have the enzyme that makes the omega-9 isomer... So, what is the pathway for formation of this unusual isomer? I am not going to get much into this, so let's just say that we did a lot of deuterium labelings and electron impact mass spec analyses, to finally come out with the pathway for 16:1n-9 synthesis, which is show on the right (**Slide 18** – Metabolic Origin of 16:1n-9? No Δ7 Desaturases in Mammals!). It's a 4-step process and actually a quite convoluted one, because it starts with palmitic acid which is subsequently, elongated to stearic, desaturated to oleic and finally β-oxidized to 16:1n-9. Now, what could be the biological meaning of all of this? Well, I believe this is telling us is that when white blood cells are being activated by a proinflammatory stimulus, they activate both de novo biosynthetic pathways, and catabolic β-oxidative pathways to generate fatty acid diversity, to produce fatty acids that could not be formed another way, such as 16:1n-9, which is then directed to lipid droplets (**Slide 19** – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism).

At this point, the burning question here is, what is this fatty acid doing in the monocytes? What is its biological role? (**Slide 20** – What Is the Biological Relevance of 16:1n-9 in Phagocytic Cells?). If you remember, I said a few minutes ago that palmitoleic acid is suggested to be anti-inflammatory; thus, we decided to assess whether 16:1n-9 could be anti-inflammatory as well. 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 21** – 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 22** – 16:1n-9 Possesses Anti-inflammatory Properties *in vitro*). As a control for these experiments we also used cells enriched in palmitoleic and 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 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; 16:1n-7 had significant effects only in two of them, Tnf and Nos2. We also conducted experiments with mice (**Slide 23** – 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, these data suggest that the relatively uncommon fatty acid acid 16:1n-9 as a novel anti-inflammatory fatty acid whose activity is clearly distinguishable from that of 16:1n-7 (**Slide 24** – 16:1n-9 as a novel anti-inflammatory fatty acid). It is quite remarkable that this fatty acid possesses unique biochemical properties, such as that it is produced on cellular demand and tends to accumulate in neutral lipids.

At the end of my talk let's get back briefly to 16:1n-10, or sapienc acid, the isomer which was found at rather low levels in monocytes. Conventional wisdom says that "sapienic acid is very abundant in human sebum, skin and nails, and among hair-bearing animals is restricted to humans"; This sentence, with slight variations, can be found in many papers, websites, even in the Wikipiedia, thus leading to the general assumption that sapienic acid is a human-only fatty acid; the name sapienic comes from sapiens, as in 'homo sapiens' (**Slide 25** – 16:1n-10: Sapienic Acid). However, quite significant levels of sapienic acid were unequivocally found in murine peritoneal macrophages (**Slide 26a** – Analysis of 16:1 Isomers in Cell Not of Human Origin). Moreover, when we analyzed RAW264.7 cells, a murine cell line which is widely used as a paradigm for studies of macrophage lipid signaling and metabolism, 16:1n-10 was detected at levels comparable to those of 16:1n-7, and much higher than those of 16:1n-9 (**Slide 26b** – Analysis of 16:1 Isomers in Cell Not of Human Origin). To study whether sapienic acid has also anti-inflmmatory activity in these cells, we prepared cells enriched in this fatty acid just as we had done before for the n-9 and n-7 isomers.

This slide shows the effect of enriching the cells with 10 μM fatty acids on gene expression (**Slide 27** – Anti-inflammatory Effect of 16:1 Fatty Acids). At 10 μM, sapienic had no effect. Dose-response experiments indicated that significantly higher concentrations of 16:1n-10 (>25 μM) were necessary to observe a similar anti-inflammatory effect (**Slide 28** – Anti-inflammatory Effect of 16:1 Fatty Acids) and, at doses at which 16:1n-10 was ineffective (<25 μM), it did not alter the anti-inflammatory effect of 16:1n-9. In another series of experiments, we used yeast zymosan instead of LPS as a proinflammatory agent and no significant effects were observed (**Slide 29** – Anti-inflammatory Effect of 16:1 Fatty Acids). Thus it seems that the anti-inflammatory effect of fatty acids is exerted specifically on LPS signaling via TLR4. And, in that sense we have data preliminary data suggesting that 10 μM 16:1n-9 blunts the LPS-induced phosporylation of MAP kinases p42/p44 in a time-dependent manner, while 16:1n-10 is ineffective (**Slide 30** – Effect of 16:1 Fatty Acids on LPS-induced Signaling). So, in conclusion, (**Slide 31** – Phagocytic Cells Express Multiple 16:1 Isomers), our results suggest the presence in phagocytic cells of an unexpected variety of 16:1 isomers which can be distinguished on the basis of their biological activity (relative potency and range of concentration), cellular regulation, and perhaps location.

But from a pathophysiological point of view, what could be the importance of phagocytic cells expressing this variety of isomers. If the bouble bonds of these isomers were to be hydrated then we would have an even wider variety of hydroxyfatty acids (**Slide 32** – Multiple Hydroxyfatty Acids Generated by 16:1 Isomers). An this is important because these hydroxyl groups could be esterified by other fatty acids, generating in this manner this novel family of compounds, which have been found to possess anti-diabetic properties (**Slide 33** – Hydroxyfatty Acids as Precursors of Novel Anti-Diabetic Acids). With different compositions, at least 16 FAHFAs have been described to date. These compunds have shown string anti-diabetic effects in animal models of metabolic disease as they help improve glucose homeostasis at different levels in different tissues and probably via different mechanisms involving various receptors (**Slide 34** – Hydroxyfatty Acids as Precursors of Novel Anti-Diabetic Acids) . Thus, in the light of these very novel data, our results describing multiple 16:1 isomers in phagocytes and their pathways of synthesis are very relevant because they may provide us with novel targets for pharmacological intervetion (**Slide 35** – Anti-inflammatory Fatty Acids 16:1n-7, 16:1n-9, 16:1n-10). Mnipulating the routes of synthesis of these compounds we might be able to improve diabetes. Just to conclude, the Acknowledgments slide (**Slide 36** – Acknowledgments). Thank you very much for your attention.

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