Phospholipase A_2-regulated Lipid Droplet Formation in Leukocytes

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This a transcription of the lecture presented on Tuesday May 21, 2013 at the 5th International Conference on Phospholipase A_2-mediated Signaling in Translational Medicine in New Orleans, LA, USA (Slide 1).

This very large cell is a human macrophage, derived from blood monocyte by continued culture in the presence of human serum. The cell is stained in blue with a protein of lipid metabolism called lipin-1, which localizes on the surface of these cytoplasmic formations in orange that tend to distribute in the periphery of the cells (Slide 2 – untitled) [1]. These formations are lipid droplets and, as you can see, macrophages have many of them. But 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). 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 one 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 $A_2\alpha$ ($cPLA_2\alpha$), cyclooxygenase-2 or lipin-1, all localize to this organelle. I will get back to $cPLA_2\alpha$ in a little while; this is the first enzyme in the eicosanoid cascasade, the one that opens the door to eicosanoid production by releasing free arachidonic acid (AA) from phpospholipids [2,3].

Free exogenous AA can induce formation of lipid droplets in human monocytes (Slide 4 – AA Induces Lipid Droplet Formation) [4]. 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. We also studied the effect of palmitic acid, a fatty acid that at much higher concentrations is proinflammatory [5]. 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 5 – AA Induces Neutral Lipid Formation) [4]. Of course, AA is a lipid (Slide 6 – AA Effects on Lipid Droplet Formation), so this elevated neutral lipid production could just occur as a consequence of a 'passive' incorporation of the fatty acid into neutral lipids. A second possibility is that AA actually activates the cells and thus neutral lipid production is the consequence of an 'active' signaling component which promotes the incorporation of other fatty acids in addition to AA. To distinguish between the two possibilities we used triacsin C, a compound that inhibits some members of the acyl-CoA synthetase family of enzymes and that, at least in monocytes, blocks the incorporation of the exogenous AA into neutral lipids (the 'passive' component) but not the incorporation of the endogenous fatty acids (the 'active' component). We took the monocytes and treated them with AA in the absence or presence of triacsin C (Slide 7 – TAG Fatty Acid Composition in Lipid Droplets) [4]. If you look on the left hand side, triacsin C blocked partially the production of TAG suggesting that the effect of AA works through both passive

and active components. Now if you look on the right, this is the fatty acid profile of TAG, from left to right, myristic, palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic acids. In the absence of triacsin C there is a huge incorporation of AA, however in the presence of the inhibitor this is totally prevented. Thus the inhibitor worked pretty nicely, but the important thing here is that no other fatty acid was affected by triacsin C; incorporation is the same whether or not triacsin C is present. So this highlights the active signaling component induced by AA and, because this is the phenomenon we are interested in and wish to characterize, from now on all the experiments include triacsin C.

I will get back to this stuff in a moment, but now please allow me to open a short parenthesis to discuss some data on the mechanism through which AA induces lipid droplets in the monocytes. In the first place, we measured the effect of AA on MAP kinases and found that AA activates p38, JNK, but not the ERKs (Slide 8 – AA Activates p38 and JNK) [4]. In accordance with these data, AA induces the phosphorylation of cPLA$_2\alpha$ (Slide 9 – AA Stimulates cPLA$_2\alpha$ Phosphorylation by both p38 and JNK) [4]. This phosphorylation is not affected by inhibitors of ERK, as expected since AA does not activate the ERKs. However, when both inhibitors are present, cPLA$_2\alpha$ phosphorylation is reduced at levels even lower than those found in unstimulated cells. Now, how does this relate with lipid droplet formation? (Slide 10 – Role of cPLA$_2\alpha$ in LD Formation) [4]. Here we have our monocytes with their nuclei stained in blue with DAPI. When treated with AA they make lipid droplets in green (BODIPY) and, in agreement with previous slides, if we block cPLA$_2\alpha$, in this case with a pretty well established inhibitor, pyrrophenone [6,7], lipid droplet formation is strongly inhibited, as expected. Now if we use the p38 inhibitor not very much happens, the cells still prodce significant numbers of lipid droplets (Slide 11 – Role of cPLA$_2\alpha$ in LD Formation) [4]. The same occurs if we use the JNK inhibitor. However, if we use both at the same time, strong inhibition of lipid droplet formation is observed. Thus the same conditions that block cPLA$_2\alpha$ phosphorylation/activation lead to inhibition of lipid droplet formation. As a summary of this data, we show this model (Slide 12 – Simultaneous Activation of p38 and JNK by AA Activates cPLA$_2\alpha$), where AA activates p38 and JNK (but not ERK) and this two kinases act on cPLA$_2\alpha$ to activate it, so that the enzyme regulates lipid droplet synthesis. Two points to consider. First is that it is possible that p38 and JNK act both on cPLA$_2\alpha$ simultenously to activate it; however, since both kinases phosphorylate cPLA$_2\alpha$ on the same residue, this sems a bit odd, We hipothesize that maybe there is an intermediate kinase that is activated by both p38 and JNK, and this kinase is the one that directly phosphorylates cPLA$_2\alpha$. We are currently working in the lab to verify whether this hipothesis is correct. Second, how is cPLA$_2\alpha$ mediating lipid droplet formation? The answer is: we do not know. We know however that cPLA$_2\alpha$ does not regulate the synthesis of neutral lipids, so we speculate that some step of the formation, the budding of the lipid droplet out of the endoplasmic reticulum may be controlled by cPLA$_2\alpha$.

This slide shows the fatty acid profile of TAG and CE esters in the presence of triacsin C (Slide 13 – Fatty Acid Content of TAG and CE) [4]. The data on the left are the same as previously shown (slide 7). The profiles are very similar in qualitative terms in both cases, and of course there is no AA because of the presence of triacsin C. The important thing in this slide is the fatty acid in the purple box: palmitoleic acid. You can see there is very little in resting cells, and that it hugely increases in activated cells, hence we suspect if 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; it has been implicated in regulating inflammation, and it has been suggested as well that this fatty acid functions as an adipokine, released by the adipose tissue to regulate lipid metabolism in liver. Thus our work adds to these results, and shows that activated monocytes synthesize palmitoleic acid and store it in significant quantities in the neutral lipids of lipid droplets.

The next question that we want to answer is, what is the origin of ths palmitoleic plus other fatty acids? (Slide 14 – Origin of the Fatty Acids – Possibilities). There are two possibilities: first is that the fatty acids come from membrane phospholipids. If this is the case, then total cellular fatty acid should remain constant. The second

possibility is that A activated de novo fatty acid synthesis. In this case, total cellular fatty acid should increase. Thus we measured total fatty acids in cells and the result is clear, there is an increase in cellular fatty acids, thus indicating that AA indeed activates fatty acid de novo synthesis to make lipid droplets (Slide 15 – Total Fatty Acid Content of Human Monocytes) [4]. On the right we have the fatty acid profile of whole cells, that is neutral lipids plus phospholipids, and still under these conditions a significant increase in palmitoleic acid levels is observed.

Four genes control fatty acid synthesis in mammalian cells (Slide 16 – Expression of Genes Involved in de novo Fatty Acid Synthesis). These are acetyl-CoA carboxylase, that makes malonyl-CoA. Malonyl-CoA is used by fatty acid synthase to make palmitic acid, which can be either elongated to stearic or desaturated to palmitoleic acid. Stearic acid can be desaturated to oleic acid by the same desaturase that makes palmitoleic acid. We measured these four genes by qPCR and found that all of them were increased by AA in monocytes (Slide 17 – Expression of Genes Involved in de novo Fatty Acid Synthesis). As a conclusion of this part of my talk (Slide 18 – Lipid Inflammatory Signals Regulate Cellular Lipid Metabolism), we believe that our data constitute an excellent example of a lipid proinflammatory signal, AA, acting on its target cell, the monocyte, to deregulate lipid metabolism, in this case increasing fatty acid synthesis. Among other things, this has the effect of increasing the cellular amount of palmitoleic acid, which can be sent to lipid droplets or exert other effects on the cells. If palmitoleic acid was proinflammatory, which in some occassions could be, it can be said that, in a way, AA is perpetuating itself, or better the proinflammatory signal, with a different face. Outside is AA, inside the cell is palmitoleic acid. Indeed, in analogy with AA, palmitoleic acid added exogenously to cells can trigger lipid droplet formation as well (Slide 19 – Palmitoleic Acid "Actively" Induces Lipid Droplet Formation…), in a $cPLA_2$-dependent manner (Slide 20 – … in a $cPLA_2$-dependent manner!)

By using GC/MS we determined next the distribution of palmitoleic acid between phospholipid classes, phosphatidylinositol, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in resting and AA-treated monocytes (Slide 21 – 16:1-Contaning Phospholipid Classes (GC/MS)). In resting cells the richest class in 16:1 is phosphatidylcholine, but this changes in the activated cells. In qualitative terms, the class that increases the most is phosphatidylinositol. There is little in resting cells but a clear increase is seen in activated cells. This fact, along with a technical reason that I will mention later, made us focus on this class of phospholipids. By using LC/MS [9-11] we separated the molecular species of phosphatidylinositol (Slide 22 – Novel 16:1-PI Species That Appear After Activation (LC/MS)). To restrict the search and obtain only a few hits, we focused only on those palmitoleic phospholipids that showed up in activated cells but did not occur in resting cells. By doing this, we got two, maybe three species. The first one contains not one but two palmitoleyl lateral chains. The second one, which increased way more than the first one after activation, contains palmitoyl and palmitoleyl chains. Finally, there is maybe a third one, which contains stearic in addition to palmitoleic. Problem with this one is that it is isobaric with this other which is a major one. Isobaric means that they both have the same mass to charge ratio, thus we cannot resolve them with our machine. Thus at this point we cannot tell how much of this increase, if any, is actually due to the palmitoleic-containing lipid. Never mind, we still have two excellent candidates for our studies. So our strategy from now on is to make these lipids in the lab, to introduce them into the cells, and see what happens. Well, for doing this it is really fortunate that these lipids are of the inositol class, because inositol lipids are anionic, and anionic lipids can be transfected into cells just like you transfect DNA or RNA [12]. This is the technical reason I was referring to before. Thus, by using lipofectamine, lipofectin, or anything on that sort, you name it, you can get the lipid inside the cells and study its effects on a number of cell functions (Slide 23 – Intracellular Delivery of Anionic Phospholipids). At this point I have to stop the palmitoleic acid story here because we still have no data to show but, please let me talk instead for the rest of my talk about an unusual phospholipid, also a PI molecule, which does not contain palmitoleic acid, but two arachidonoyl tails (Slide 24 – 1,2-Diarachidonoyl-sn-glycero-3-phosphoinositol), because we believe this lipid may be involved in the regulation of innate immune responses in macrophages [11]. These experiments were conducted in murine macrophages so from now to the end of the talk I will refer to these cells.

In this slide there is the full profile of AA-containing phospholipids of murine macrophages (Slide 25 – AA-Containing Phospholipids in Resting Macrophages) [11]. In red there are the choline phospholipids, in green the etanolamine phospholipids, in yellow the inositol lipids, and in pink the serine phospholipids. By class on the right, the richest class is the green, followed by the red. Lesser amounts in yellow and pink. Well what happens when we stimulate the cells? Of course AA will be liberated and the amount of these species will decrease. This is what we have in this slide (Slide 26 – AA-Containing Phospholipids in Zymosan-Stimulated Macrophages) [11]. Most of the red phospholipids decrease significantly, as it does one of the yellow ones. The pink vary little and the green do not change at all. But this is well described so no point here. The point is, you see two lipids that actually increase, not decrease. The first one, red, is PC(20:4/20:4), but again this is no new, as this phospholipid was described some 25 years ago. However a second one, the inositol equivalent, PI(20:4/20:4) also increases and we believe this is really new stuff [9, 11, 13]. So we proceeded to characterize it. This lipid increases linearly with time after zymosan stimulation and tends to stay elevated, a behavior that is compatible with it playing a role (Slide 27 – Stimulated Production of PI(20:4/20:4) in Macrophages) [11]. We prepared the lipid in the lab and introduced it in cells using the strategy I described previously (Slide 28 – Incorporation of PI(20:4/20:4) Into Cells). We made the complexes, gave them to the cells, waited, stimulated with zymosan and looked for responses. Initially we focused on gene expression, because that is the most "fashionable" response one can measure, right? So we stimulated the cells, either untreated or loaded with PI(20:4/20:4) and measured the expression of various genes by qPCR (Slide 29 – PI(20:4/20:4) Does Not Regulate Gene Expression) [11]. Zymosan induced significant increases which were the same in control and in PI(20:4/20:4)-loaded cells. Also, the lipid did not do anything on its own. So it is clear that PI(20:4/20:4) does not regulate gene expression, which was quite a disappointing finding. However it got us thinking that perhaps we would have better look at short-term, acute responses. And among these, what a better response to measure than production of reactive oxygen intermediates, superoxide anion in this case? (Slide 30 – PI(20:4/20:4) Regulates Superoxide Anion Production) [11]. We stimulated the macrophages with either PMA or zymosan and obtained nice responses, which were significantly increased when PI(20:4/20:4)-loaded cells were used. Granted, the increases are not very impressive; however, when we used cells loaded with an irrelevant lipid, no increase was appreciated. More importantly, when we assayed another immediate response, that is, secretion of lysosomal hydrolases lysozyme, we observed again a significantly increased response when PI(20:4/20:4)-loaded cells were used (Slide 31 – PI(20:4/20:4) Regulates Lysozyme Release).

So, as a conclusion of my talk, and this is my last slide (Slide 32 – Novel Lipid Mediators of Macrophage Activation), we have described novel lipid mediators of phagocyte activation, palmitoleic acid and PI(20:4/20:4), and these two lipids appear to be oproducts pf phospholipase A_2-mediated signaling. The fun starts now in the lab, as we have to define pathways and effectors impacted upon by these mediators. To conclude, I would like to thank all the people in my lab who have been involved in these projects, and to my collaborators, Dr. M. Balboa from my institute, and Dr. E. Claro from the Autonomous University of Barcelona, and also to our sponsors, for as long as they last (Slide 33 – Acknowledgments). And I thank you very much for your attention.

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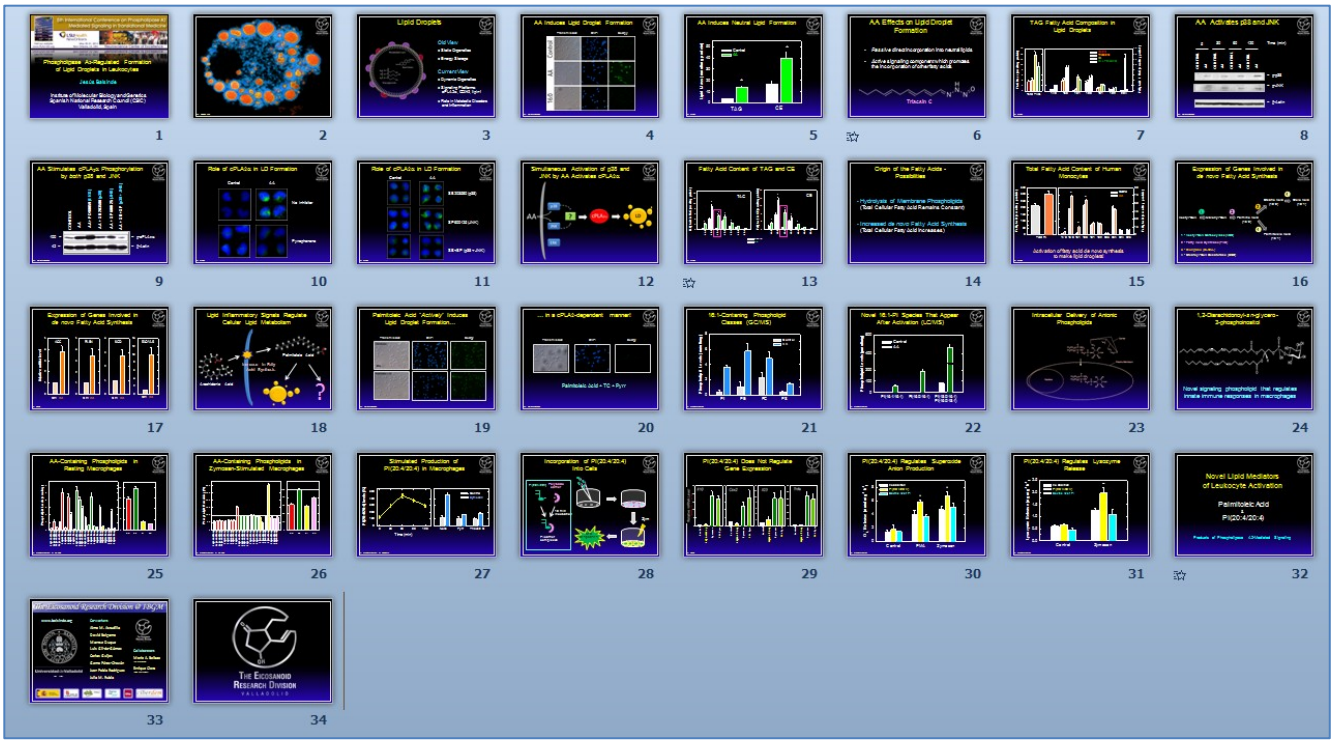
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The Eicosanoid Research Division
Valladolid