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POTENTIATION OF MITOCHONDRIAL ACTIVITY IN ADIPOSE TISSUE AS A THERAPEUTICAL STRATEGY FOR OBESITY AND TYPE 2 DIABETES: INTERPLAY BETWEEN OXYGEN SENSING AND NEUREGULIN SIGNALLING

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1. Summary of the project

There is emerging interest in understanding white adipocyte mitochondrial dysfunction in obesity driven metabolic disease because leads to adipocyte enlargement but also pathological adipokine secretion. This project aims to unveil the mechanisms involved in this mitochondrial dysfunction to design strategies to restore WAT mitochondrial activity and combat pathological WAT expansion. Insufficient oxygen supply to white adipocytes during WAT expansion activates hypoxia-inducible factors (HIFs)-dependent mitochondrial dysfunction but the underlying HIF-dependent mechanisms still remain elusive. Our data indicates that hypoxic conditions leading to HIF1a activation may counteract WAT neuregulin (NRG) signaling through the expression of the NRG co-receptor ErbB1-feedback inhibitor ERRFI1. Moreover, the reduced levels of a recently identified novel adipokine, NRG4, together with the promitochondrial properties of NRG /ErbB pathway in other settings, led us to propose a role of neuregulin in healthy WAT biology, which can be compromised by pathological HIF1a-driven WAT expansion. However, our data have not revealed a higher expression of ERRFi1 in white adipose tissue in obesity conditions. Surprisingly, we found that the activation of the hypoxia-inducible factor HIF2 promotes the expression of NRG4 defining a new therapeutic axis HIF2-NRG4 that could be pharmacologically potentiated. Using a combined experimental approach of human samples, novel mouse transgenic models and mitochondria molecular analysis, we first propose to analyze WAT-derived NRGs, ErbB/HER receptors and NRG signaling regulators that are affected in obese and diabetic subjects. Secondly, we propose to identify those NRGs that could potentiate white adipocyte mitochondrial activity, assembly of mitochondrial complexes, and substrate oxidation in order to counteract WAT mitochondrial dysfunction in vivo. Importantly these approaches are operational for this project and go beyond what is normally employed in other studies of WAT biology because current approaches for adipocyte restricted gene inactivation cannot discriminate between white and brown adipose tissue. Collectively, this project provides novel understanding in mitochondrial dysfunction in WAT through repression of NRG signaling and opens novel therapeutic avenues to restore healthy WAT mitochondrial metabolism in obese patients.

2. Results of the project

Neuregulin (NRG4) is an adipokine with multiple protective effects either local on the adipocytes and distal on the hepatocytes, whose effects antagonize with the induction of insulin resistance, based on preliminary bibliography data and those obtained in this project, which are summarized in the following contributions:

1- Findings in human study

1.1. Neuregulin 4 Is a Novel Marker of Beige Adipocyte Precursor Cells in Human Adipose Tissue

In mice, neuregulin 4 (NRG4) expression has been linked to brown adipose tissue activity and browning of white adipocytes. Here, we found in humans that both subcutaneous (SAT) and visceral (VAT) adipose tissue *NRG4* gene expression was positively correlated with markers of brown/beige (*UCP1, UCP3, and TMEM26*) adipocytes. In addition, SAT *NRG4* expression was associated with insulin action, whereas VAT *NRG4* expression was negatively correlated with expression of lipogenic and proinflammatory genes. To sum up, current findings suggest *NRG4* gene expression as a novel marker of beige adipocytes in human adipose tissue.

1.2. Serum NRG4 is not associated to obesity and its associated metabolic disturbances, including insulin resistance, in non-diabetic subjects.

In humans, the association between circulating NRG4 and obesity-associated insulin resistance and liver steatosis is less clear due to some contradictions in previous studies. In the present study we examined the potential relationship between circulating NRG4 and obesity-associated metabolic disturbances in non-diabetic subjects in two independent cohorts. No significant associations were found between serum NRG4 and obesity, insulin resistance or liver steatosis in either cohort.

1.3. Adipose tissue epidermal growth factor receptor impacts on human adipogenesis.

To clarify the potential role of EGFR and human ErbB receptor (HER) 2 on human adipogenesis and on human adipose tissue, and to study the potential role of other HERs receptors and neuregulins in this tissue, in the present project the expression of HER receptors and neuregulins in human subcutaneous and visceral adipose tissue according to obesity and insulin resistance were investigated. Main findings:

- *HER2* and *HER4* mRNA were increased in obesity without any associations with insulin resistance, hyperglycemia or dyslipemia. Otherwise, SAT *EGFR* mRNA, which did not change according to obesity, was negatively correlated with insulin resistance.
- Of note, both SAT and VAT *EGFR* mRNA were positively correlated with expression of some adipogenic genes (including *ADIPOQ*, *SLC2A4*, *FSP27, PLIN* and *PPARG*).
- In line with these observations, *in vitro* experiments indicated that *EGFR* gene knockdown impaired adipogenesis during the early stage of adipocyte differentiation in human subcutaneous preadipocytes and in fully differentiated human subcutaneous adipocytes.
- Another important finding of this study was association between neuregulin 2 (NRG2) expression and adipogenesis in human adipose tissue.

2- Role of the neuregulin 4 (NRG4) in the adipocyte biology and possible systemic effects

2.1. NRG4 is expressed by adipocytes but not by the stromal fraction of the adipose tissue and by adipocytes cultures both primary and the stable cell line 3T3-L1. The silencing of the expression of NRG4 or its ErbB receptors alter the adipocyte biology and the insulin sensitivity.

The data provided at the beginning of the project allowed us to observe that the adipocytes (from the isolation of the adipose fraction of the mice adipose tissue, visceral and subcutaneous, as well as from primary cultures of such adipocytes and in the stable cell line 3T3-L1) express NRG4 and its specific receptor ErbB4, as the coreceptors ErbB1 and ErbB2. From this it was deduced that there must be local effects of the adipokine NRG4 on the adipose tissue itself. We obtained silencing models for the expression of NRG4, ErbB4 and ErbB2 in primary adipocytes cultures and transformed adipocytes 3T3-L1. The silencing of NRG4 and the one of its receptor ErbB4 showed similar characters but the silencing of ErbB2 triggered a disruption of the whole adipogenic process. We centered on the characterization of the NRG4 silencing model in 3T3-L1 adipocytes. The loss of NRG4 triggered a total insulin resistance on its capacity to induce glucose transport.

2.2. The silencing of the NRG4 expression triggers the induction of proinflammatory characters that drive to the repression of the insulin receptor expression.

With the lack of NRG4, the expression of the insulin receptor is reduced. We observed that in these adipocytes the NFkB pathway is induced that leads to an overexpression, late in the adipogenesis, of proinflammatory cytokines such as TNFa, IL1b and IL6. Anti-inflammatory agents such as dexamethasone or salicylate made it possible toreverse the expression of proinflammatory cytokines and recover the expression of the insulin receptor.

2.3. The anti-inflammatory character of NRG4 is also manifested on macrophages.
Macrophages (M0), both primary (of the mouse bone marrow) or transformed (RAW), induced to an M1 differentiation of proinflammatory character, were submitted to conditioned media obtained from control 3T3-L1 adipocyte cultures, rich in NRG4. Both a pro-inflammatory reversal effect as well as a preventive effect, when macrophages received the treatment during the differentiation process, were observed. Given that tissue macrophages are the main producers of pro-inflammatory cytokines in adipose tissue under obesity and diabetes, this result points to a local protective effect of NRG4 against inflammation. In this sense, it should be noted that macrophages express high levels of the ErbB4 receptor.

2.4. The silencing in the NRG4 expression promotes autophagic degradation of vesicles rich in the insulin-sensitive glucose transporter, GLUT4.
Adipocytes with loss of NRG4 expression show a dramatic drop in the protein content (but not in the mRNA) of the insulin-sensitive GLUT4 transporter, as well as in the protein content of other markers of GLUT4-storage vesicles such as IRAP and Syntaxin-6. By using the functional disruptor of lysosomes, bafilomycin A1, which allows the arrest of the autophagic process, it was possible to establish that the adipocytes silenced for NRG4 presented a higher autophagic flow and that after they were stopped, the levels of the GLUT4 transporter could be recovered.

2.5. The silencing of NRG4 triggers oxidative stress and an altered morphology and functional mitochondria in 3T3-L1 adipocytes.
The causes of the induction of inflammatory characters, as well as autophagy, could well be found in an alteration of the mitochondria. In this sense, we have described that the 3T3-L1 adipocytes silenced for the expression of NRG4 have a high production of superoxides (ROS). A high portion of them are produced by the mitochondria. A slight loss in total mitochondrial content was observed and the remaining mitochondria

showed morphological characters associated with mitochondrial stress, small and rounded with loss of the ability to form filamentous structures. In addition, they showed an increase in the membrane potential and a loss in the uncoupling protein UCP1, which could contribute to the generation of superoxides. Likewise, an increase in TAG lipolysis, typical of inflammatory conditions, and the loss of insulin action on glucose oxidation and lipogenesis was observed. The study of metabolic intermediates of the Krebs cycle such as citrate, malate, succinate, and fumarate, suggested a reduction in the rates of functioning of this metabolic pathway, at the same time as a high production of branched chain amino acids was observed, which have been associated with situations of insulin resistance. All of this is consistent with the existence of mitochondrial metabolic stress.

2.6. The adipocytes silenced for the expression of NRG4 show increases in the expression of HIf1α and a loss in the expression of the vascular endothelial growth factor (VEGF).

During the project, studies were published indicating that NRG4 was an adipokine with angiogenic effects. Our studies indicated that in a situation of inducing cellular stress with DMOG, which reproduces the effects generated by hypoxia, the expression of NRG4 is increased in control adipocytes, pointing to a protective role of NRG4. In this sense, we have found that adipocytes silenced for NRG4 increase HIF1α levels and lose the ability to express the angiogenic factor VEGF.

2.7. Systemic effects of NRG4: blockage of ErbB4 expression in the skeletal muscle.

Studies in skeletal muscle-conditioned ErbB4 knockout mice, a tissue that had not yet been addressed in the literature, allowed us to study the possible existence of a distal communication between adipose tissue and skeletal muscle, the main glucose uptake tissue under absorptive conditions. Although there appear to be compensatory effects at the level of glucose and insulin tolerance responses, we were able to determine that skeletal muscle, as happened with adipocytes, shows an increase in proinflammatory cytokines, as well as in FGF21, a myokine that is induced by mitochondrial stress. Under these conditions the liver shows a loss in the ability to produce NRG4, which seems to be compensated by the production in white adipose tissue, since neither liver nor WAT shows an induction of inflammation. The studies that are detailed in the last year's scientific report point to the existence of tissue communication axes, mediated by NRG4 and its receptor ErbB4, between the muscle and the liver, the latter acting as

a mediator in the communication between WAT and muscle. Future studies should go deeper into these tissue communication axes and its impact on insulin resistance.

3- Role of hypoxia in the regulation of the NRG-ErbB1 system.

The hypoxia factors HIF1 or HIF2 are central regulators of cell autonomic metabolism. The signaling mediated by neuregulins and its ErbBs receptors are also involved in pathophysiological scenarios such as obesity or insulin resistance. However, the role of HIF factors in signaling mediated by neuregulins and its ErbBs receptors remains unknown. Our data has revealed:

3.1. Hypoxia induces the expression of ERRFi1 in in vitro adipocyte cell models but not in white adipose tissue in obesity.

Our data have revealed that ERRFi1, an inhibitor of ErbBs signaling, is induced in 3T3-L1 cells (preadipocyte cell line) exposed to hypoxia. However, this positive regulation of ERRFi1 is not manifested in adipose tissue of mice fed with high-fat diet, which triggers the activation of HIF1a. These data may suggest that HIF1a activation *in vivo* might occur less markedly than 3T3-L1 cells exposed to hypoxia. Alternatively, it may be possible that this HIF1a-ERRFi1 pathway is not operative *in vivo* during expansion of white adipose tissue.

3.2. The hypoxia-inducible factor HIF2a induces NRG4 in the liver and white adipose tissue.

Using animal models of gain and loss of function of the HIF factors (using the Ubc-CreERT2 system), we first identified that the expression of NRG4 is highly induced in response to HIF2 isoform activation in the liver. These data led us to shift our attention from ERRF1 to NRG4. Along these lines, previous data from other groups have shown that moderate activation of HIF2a in liver tissue facilitates insulin sensitivity and metabolic dysfunction. Therefore, we are currently considering that NRG4 might mediate this beneficial effect of HIF2a activation on liver metabolism. Furthermore, we have recently discovered that, similar to liver tissue, potentiation of the HIF2 isoform markedly induces NRG4 in white adipose tissue. Therefore, the beneficial effects in obesity mediated by NRG4 could be induced by the pharmacological activation of HIF2a (see also below).

3.3. The proximal promoter of NRG4 presents consensus binding sites for the binding of HIF2a.

The data presented in section 3.2 led us to analyze the presence of possible DNA binding sites for HIF in the mouse and human NRG4 promoter. In this line, we identified three RCGTG motifs in the proximal promoter of the NRG4 promoter, both human and mouse, in position 5' of exon1 of NRG4. Importantly, these three possible HIF binding sites are in regions characterized by histone 3 modifications such as H3K4Me1, H3K5Me3, or H3K27Ac, which are commonly found in regions of active transcription.

3. Clinical implications and future research lines

Insulin resistance involves a lower range chronic inflammation in adipose tissue. Current study led us to conclude that:

1. Adipokine neuregulin 4 is an endocrine factor with anti-inflammatory effects on adipocytes based on the models generated for this project of loss of function
2. Neuregulin 4 treatment can reverse inflammation characters in adipocytes
3. Neuregulin 4 preserved macrophages of inducing a pro-inflammatory profile
4. Neuregulin 4 preserves insulin sensitivity by exerting a control over inflammation and autophagic flow, which ensures the expression levels of the insulin receptor and the insulin-sensitive glucose transporter GLUT4 in adipocytes
5. The lack of neuregulin 4 leads to mitochondria dysfunction and superoxide production in adipocytes
6. Studies in vivo indicate that the lack of action of Neuregulin 4 in skeletal muscle, induces muscle inflammation and mitochondrial dysfunction based on the expression of FGF21.
7. There is a cross-talk between muscle and liver in terms of sustaining the hepatic neuregulin 4 expression
8. Regarding the regulation of NRG4 by the HIF2a isoform, it should be considered that (i) the pharmacological activation of the HIF pathway by using PHD oxygen sensor inhibitors combats the signs of metabolic syndrome in mice and (ii) moreover it has been shown that NRG4 has multiple metabolic benefits. Therefore, these data suggest

that pharmacological activation of the HIF2 system, by PHD inhibitors, may represent a new therapeutic approach to induce beneficial metabolic effects mediated by NRG4.
9. In human adipose tissue, neuregulin 4 expression is a new marker of thermogenic/beige cells. This finding indicates that the induction of NRG4 in human adipose tissue might enhance energy expenditure, and in consequence, reduce excessive fat mass accumulation in subjects with obesity.
10. Current study also confirms the relevance of EGFR in adipose tissue adipogenesis and demonstrates the association of HER2 and HER4 with obesity.

These findings suggest a new therapeutic approach targeted to enhancing EGFR and silencing HER2 and HER4 receptors to improve obesity-associated metabolic disturbances.

As a whole, neuregulin 4 – ErbB4/HER4 receptors are essential to sustain insulin sensitivity in adipocytes and prevent inflammation, and they also have an endocrine role targeting skeletal muscle and liver with systemic consequences that should be addressed more profoundly in the future. With this in mind, neuregulin 4 emerges as a potential therapeutic tool to prevent inflammation and insulin resistance. In this sense, the search of pharmacological agonists specific for the neuregulin 4 receptor HER4, could be a novel alternative to treat obesity-related type 2 diabetic patients.

4. Scientific bibliography generated

1. Neuregulin 4 Is a Novel Marker of Beige Adipocyte Precursor Cells in Human Adipose Tissue. Comas F, Martínez C, Sabater M, Ortega F, Latorre J, Díaz-Sáez F, Aragonés J, Camps M, Gumà A, Ricart W, Fernández-Real JM, Moreno-Navarrete JM. Front Physiol. 2019 Jan 31;10:39. doi: 10.3389/fphys.2019.00039. eCollection 2019.

2. Neuregulin, an effector on mitochondria metabolism that preserves insulin sensitivity. Gumà, A., Díaz-Sáez, F., Camps, M., Zorzano, A. Frontiers in Physiology, 11, art. 696, 2020. REVIEW. doi: 10.3389/fphys.2020.00696

3. Neuregulin 4 downregulation induces insulin resistance in 3T3-L1 adipocytes through inflammation and autophagic degradation of GLUT4 vesicles. Francisco Díaz-Sáez, Carla Blanco, Adrià Archilla, Xavier Testar, Maria Isabel Hernández-Alvarez, Sílvia Mora, David Sebastian, Montserrat Romero, Wifredo Ricart, José Manuel Fernández-Real, José María Moreno-Navarrete, Julián Aragonés, Antonio Zorzano, Marta Camps, Anna Gumà. *Submitted to Diabetologia, International peer-review Journal, IF 7,1*

4. **Adipose tissue epidermal growth factor receptor impacts on human adipogenesis.** Latorre J, Martínez C, Ortega F, Lluch A, Oliveras-Cañellas N, Díaz-Sáez F, Aragonés J, Camps M, Gumà A, Ricart W, José Manuel Fernández-Real JM, Moreno-Navarrete JM. *In process*

5. **Serum NRG4 is not associated to obesity and its associated metabolic disturbances.** Martínez C, Latorre J, Ortega F, Lluch A, Oliveras-Cañellas N, Aragonés J, Camps M, Gumà A, Ricart W, José Manuel Fernández-Real JM, Moreno-Navarrete JM. *In process*