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CONTROL OF SYSTEMIC AND TISSUE-SPECIFIC OBESITY-INDUCED INSULIN RESISTANCE BY NUCLEAR RECEPTORS

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1. Summary

A chronic low-grade inflammatory response (known as metaflammation) is considered a major etiologic factor for obesity-associated insulin resistance, an early trait in the development of type 2 diabetes. Accordingly, activation of pro-inflammatory pathways by inflammatory cytokines and lipid metabolites interferes with insulin receptor signaling, thereby promoting insulin resistance. Members of the peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and retinoid acid receptor (RXR) subfamilies of nuclear receptors (NRs) are activated by specific agonists and, in turn, play central roles in carbohydrate and lipid metabolism as well as in the modulation of immune responses. In this sense, through their capacity to crosstalk with pro-inflammatory pathways, these NRs exert anti-inflammatory and insulin-sensitizing activities that are potentially relevant for the pharmacological actions of their ligands, although the mechanisms underlying these activities are not fully understood.

To study different aspects related to the mechanisms and actions of PPARγ, LXRs and RXRs in obesity-associated insulin resistance we took advantage of a broad set of transgenic mouse models, which included genetically deficient mice (knockout, KO) for some of these NRs, such as macrophage-specific KO for *Rxrα* and *Rxrβ* (Mac-RXR-KO), deficient in *Rxrα*, *Rxrβ* and *Rxrγ* genes in adult cardiomyocytes (TKO), and global LXRα/β-KO and PPARγ-KO; or for selective target genes of these NRs. In addition, we used mouse models with exacerbated pro-inflammatory pathways, such as mitogen-activated protein kinase phosphatase (MKP)-1-KO mice or transgenic mice with conditional overactivation of the c-Jun N-terminal kinase (JNK) pro-inflammatory pathway specifically in macrophages (LysM-MKK7D) or in pancreatic insulin secreting cells (MKK7D), or models with defective pro-inflammatory signaling.

We demonstrated that JNK inhibition is a common trait of this set of NRs, thus expanding the list of members of the NR superfamily able to negatively crosstalk with this pro-inflammatory pathway. This action is important due to the important role of JNK activation in obesity-associated insulin resistance and type 2 diabetes. In this regard, our studies demonstrated that constitutive activation of JNK in insulin secreting cells blocks postprandial insulin release and regulates local and systemic obesity-associated insulin resistance and hyperinsulinemia.

Collectively, we have also obtained meaningful insights into the molecular mechanisms mediating the insulin-sensitizing actions of LXRs and RXRs. In addition, studies exploring the relevance of RXR transcriptional circuits in macrophages and in the adult heart reveal this transcription factor as a master orchestrator of macrophage identity, lipid homeostasis and inflammation in macrophages and cardiomyocytes. Our findings suggest that macrophage RXR is a key transcription factor controlling insulin resistance, and a very promising mediator of insulin resistance-related cardiac disease.

Overall, these studies open novel avenues to pharmacologically alleviate pathological conditions associated to obesity and type 2 diabetes, which are major worldwide epidemics.

2. Results

Characterization of novel crosstalk mechanisms between NRs and pro-inflammatory signaling. Implications in obesity-associated insulin-resistance. We have focused on the crosstalk of several NRs with the JNK pro-inflammatory pathway, and on the consequences of tissue-specific activation of this pathway in obesity-associated insulin resistance. For these studies we used two transgenic mouse models generated by our group (LysM-MKK7D and MKK7D for the activation of JNK in myeloid and pancreatic β-cells, respectively).

We had previously demonstrated that PPARγ ligands, such as the insulin-sensitizing drugs from the thiazolidinedione (TZD) family, inhibit obesity-induced JNK activation. This action is required for the insulin-sensitizing properties of PPARγ and its ligands. In this project, we have further extended the knowledge in this field by showing that LXRs and RXRs are also able to inhibit the activation of the JNK pathway. At the molecular level, both PPARγ and LXR agonists upregulated the expression of a promising candidate, the serine/threonine-protein phosphatase 5C (Ppp5C). However, Ppp5C-KO mice displayed normal levels of obesity and insulin resistance in response to a high fat diet (HFD) in comparison to control mice, and this phosphatase did not mediate the insulin sensitizing actions of PPARγ or LXR agonists. Moreover, we demonstrated that the insulin sensitizing action of PPARγ and its ligands, the TZDs, is not mediated by the

anti-inflammatory gene MKP-1. Finally, we have identified an additional PPARγ and LXR target gene that inhibits the activation of the JNK pathway in response to endoplasmic reticulum stress and inflammatory stimuli and is, therefore, a candidate to mediate in the insulin sensitizing actions of these NRs and their agonists.

Regarding the activation of the JNK pro-inflammatory pathway in specific tissues, using the LysM-MKK7D mouse model we showed that JNK activation in myeloid cells is blunted by the concomitant induction of a negative feedback mechanism, a relevant response that should be taken in account in future studies. On the other hand, from the study of the MKK7D mice we learned that activation of the JNK pathway in insulin secreting cells strongly regulates systemic insulin resistance and the hyperinsulinemia associated to obesity.

A second line of research focused on the crosstalk between LXRs, in particular, and pro-inflammatory signalling by the cytokine interferon gamma (IFNγ). We observed that the transcription factor Interferon Regulatory Factor 1 (IRF1) is required for the induction of a number of inflammatory genes that are classical mediators of the macrophage response to IFNγ. LXR activity was able to downregulate the induction of a subset of these genes without interfering with IRF1 expression. Conversely, the use of IRF1KO macrophages revealed that negative crosstalk between LXRs and IRF1 is important for the repression of a subset of LXR target genes. In contrast to the general negative crosstalk between LXRs and inflammatory signalling, we have also observed that several inflammatory mediators cooperate with LXRs for the induction of a discrete subset of LXR target genes, including CD38, a multifunctional enzyme that regulates the intracellular levels of NAD. The molecular mechanisms involved in this cooperation have been partially characterized.

Novel mechanisms involved in the insulin-sensitizing actions of NRs.

We have characterized the functional impact of RXR in insulin resistance, focusing on the study of peripheral metabolic tissues and the crosstalk between organs. To address the role of macrophage RXR in the control of lipid metabolism and inflammatory responses, we generated the Mac-RXR-KO mouse model and demonstrated that RXR is a required transcription factor for the maintenance and identity of tissue resident macrophages. We found that *RXRα* and *RXRβ* deficiency specifically in macrophages results in glucose intolerance and insulin resistance in diet-induced obese mice.

We also characterized the regulatory mechanism by which RXR controls inflammatory responses in macrophages and concluded that RXR and PPARγ are mutually required for transrepression of pro-inflammatory genes both *in vivo* and *in vitro*. Our *in vivo* findings confirm that RXR-dependent transcriptional changes in macrophages are required for the insulin-sensitizing action of RXR and PPARγ ligands. In addition, we showed that both RXR and PPARγ are required for proper transcriptional regulation of genes relevant to lipid homeostasis. In order to deeply phenotype the metabolic state of sorted macrophages, we have performed metabolome studies on mouse peritoneal macrophages. Our findings provide evidence of FACS-induced biochemical changes in the isolated cells.

To elucidate the functional contribution of cardiac RXR in the context of systemic insulin resistance and diabetic cardiomyopathy, we applied the combinatorial use of loss-of-function mouse models, advance imaging techniques and genome-wide approaches. We proved that the simultaneous absence of *Rxr*α, *Rxr*β and *Rxr*γ genes in adult cardiomyocytes (TKO) leads to a pathological phenotype, which consisted in dilated cardiomyopathy along with a decrease in systolic function. The lack of abnormalities when only *Rxr*α or *Rxr*α*-Rxrb* were deleted confirmed the existence of a redundant effect among isoforms, which enhances our molecular understanding of RXR transcriptional biology. Given the fact that diabetic patients usually present left ventricle enlargement and substantial reduction in ejection fraction, we propose the TKO model as a suitable approach to uncover RXR-mediated mechanisms in diabetic cardiac pathophysiology. We discovered that TKO hearts develop interstitial fibrosis due to the transcriptional upregulation of fibrotic genes (*Col1a1, Col1a2, Col6a2, Loxl1 etc.)* and a decrease in glucose uptake, as assessed by FDG PET-CT. Metabolic disarrangement was further evidenced by mitochondrial hyperproliferation as well as by the profound disorganization of mitochondrial structure. The fact that diet-induced obesity worsened the severity of cardiac dysfunction in TKO mice hinted for defective lipid metabolism as the putative cause of the disorder. In addition, we robustly proved that the absence of myocardial RXR results in a global dampening of the expression of genes involved in fatty acid oxidation. This transcriptional phenotype was accompanied with parallel alterations in the expression profile of genes regulating glucose and amino acid metabolism. We concluded that TKO hearts were unable to properly use fatty acids to generate ATP. Instead, an abnormal metabolic rewiring led to an enhanced oxidation of glucose, lactate and amino acids to meet the energy demands of the adult

cardiomyocyte. Collectively, our results propose RXRs as key transcriptional regulators of cellular metabolism, having functional implications in glucose balance and cardiac disease.

On the other hand, the relevance of a novel LXR target gene in the insulin-sensitizing actions of LXR agonists was evaluated in a model of HFD-induced obesity. In obese wildtype mice, treatment with an LXR agonist reduced body weight, decreased basal pancreatic insulin content and both basal and glucose-induced insulinemia, and resulted in an improvement in whole body insulin sensitivity, which was corroborated by the restoration of the capacity of the skeletal muscle to induce AKT phosphorylation during the response *in vivo* to insulin. In comparison to control mice, the mice deficient for this novel factor displayed lower systemic and pancreatic levels of insulin, and were more intolerant to glucose and more sensitive to insulin when subjected to a HFD, suggesting that both the peripheral demand and the pancreatic insulin content were reduced. Importantly, however, the protective effects of the LXR agonist on whole body insulin sensitivity and on insulin resistance in the skeletal muscle were attenuated in these mice. The actions mediated by the LXR pathway were consistent with predominant effects in insulin peripheral target tissues, including the reduction in the levels of leptin in several anatomic locations and in the expression of a subset of mediators of metaflammation in the white adipose tissue of obese mice.

3. Relevance and future implications

Some of the results we have obtained may impact the current pharmacological management of patients with type 2 diabetes. In this regard, we have shown that the activation of the JNK pro-inflammatory pathway in insulin secreting cells blocks postprandial insulin release and regulates systemic obesity-associated insulin resistance and hyperinsulinemia, thus opening a novel avenue to pharmacologically alleviate these pathological conditions. According to our data, drugs with insulin-sensitizing activity would be the most effective and recommended therapeutic tools for the treatment of obesity-associated insulin resistance and type 2 diabetes. In this sense, PPARγ ligands, such TZDs, had been applied in the clinics until side effects strongly blunted their use. Nonetheless, several clinical trials are currently in progress and novel ligands for this NR are being tested. In this project, we have obtained

evidence that ligands for other NRs, such as LXRs and RXRs, also inhibit the activation of the JNK pathway and show insulin-sensitizing action through novel mechanisms. Therefore, LXR and RXR agonists or drugs targeting specific target genes of these NRs may be alternative therapeutic tools to alleviate insulin resistance in obesity and type 2 diabetes. In particular, the feasibility of modulating RXR with pharmacological ligands (some of them already in clinical use, e.g., bexarotene) will possibly facilitate the translation of this basic research into the clinics in the medium term. We have also proven a causative relation between RXR function and the development of diabetic cardiomyopathy. Then, activation of RXR-specific circuits by pharmacological administration of bexarotene may result in effective therapies to alleviate metabolic cardiac condition in diabetic patients. Future experiments will address the suitability of bexarotene as an effective treatment against type 2 diabetes pathophysiology.

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