Final scientific report pertinent to NKFI FK 124038

Cannabinoid mediated modulation of leptin signalling

Metabolic syndrome, obesity and their complications are a global health threat (1). Despite the progress in our understanding of metabolism on the molecular level, available treatments for the above conditions have poor long term efficiency (2). Therefore, further extensive research into the regulation of metabolism remains imperative.

Both leptin and endocannabinoids are at the hub of controlling energy metabolism and they have, in general, opposing effects – whereas leptin reduces food intake and increases energy expenditure (3), the endocannabinoid system mostly supports food intake and energy conservation (4). Importantly, in obesity, the effects of leptin are supressed ('leptin resistance'; (5)) and the endocannabinoid system is overactive in almost all tissues studied (6). Our original observation showing that endocannabinoids suppress leptin signalling in the hypothalamus of healthy mice, may therefore be an important link between the overactive endocannabinoid system and the loss of leptin's biological effect in metabolic diseases. As two phenomena are now known to initiate and maintain pathological processes in obesity and metabolic syndrome (7, 8), the project aimed to clarify the molecular mechanism by which endocannabinoids curtail leptin signalling and thereby possibly uncover early steps of leptin resistance.

First, we sought to establish a *cellular* system in which to replicate the cannabinoid mediated inhibition of leptin signalling that we observed *in vivo* in mice, now *in vitro*. To this end, we expressed CB_1Rs in highly-differentiated immortalized hypothalamic neurons (GT1-7 cells) or expressed leptin receptors in Neuro 2a neuroblasts that possess endogenous CB_1Rs. By measuring leptin-evoked STAT3 phosphorylation with immunoblotting, we established in these cellular systems that i) similarly to the *in vivo* setting, leptin-induced STAT3 phosphorylation is attenuated by selective CB_1R agonists ii) the inhibitory action of cannabinoids is sensitive to CB_1R inverse agonists (rimonabant, AM 251) and to CB_1R knock-down iii) selective inhibitors of CB_2Rs do not modify the cannabinoid effect on leptin signalling iv) no significant change in the expression of the

leptin receptor (ObR_b) and suppressor of cytokine signaling 3 (SOCS-3, classic antagonist of STAT signalling; (9)) is observed upon CB_1R activation in our paradigms. Thus, we showed that cannabinoids curb leptin's effect via CB_1Rs, as opposed to other possible cannabinoid targets, and without changing leptin receptor or SOCS-3 expression.

Next, we considered alternative approaches to replace time and labour intensive western blotting for the measurement of leptin signalling. We first established that the leptin induced translocation of GFP-tagged STAT3 from the cytosol to the nucleus is a reliable measure of STAT3 activity in neurons. Then, we adapted this assay to 24 or 96-well plates and laser confocal microscopy. The resulting approach proved to be a highly sensitive, quasi-automated and high-throughput measurement of leptin-induced STAT activation. Applying this microscopic method, we demonstrated that i) CB_1Rs suppress leptin-induced nuclear translocation of STAT3 ii) pertussis toxin (PTX), inhibitor of receptor-G_i protein coupling, does not extinguish the cannabinoid effect on leptin signalling iii) inhibitors of CB_1R activated kinases, such as p42/44 ERK, p38 MAPK and JNK, do not alter CB_1R's ability to interfere with STAT activity. (We confirmed all of these observations with western blotting as well.) Thus, CB_1R mediated inhibition of leptin signalling proved to be both PTX and MAP kinase insensitive.

It has to be noted here that, unlike cytoplasmic cAMP, *mitochondrial* cAMP signalling was shown to be PTX-insensitive (10), and it has been recently recognized that cannabinoids may modulate *mitochondrial* cAMP in neurons (11). Therefore, we created mitochondria-targeted wild-type and enzymatically inactive mutant adenylyl cyclase constructs. Although manipulating *intramitochondrial* cAMP with these constructs did not interfere with cannabinoids' effect on leptin signalling, we were able to demonstrate significant augmentation of steroid synthesis by mitochondrial cAMP using the aforementioned adenylyl cyclase constructs (12).

We next turned our attention towards β-arrestins, as these signalling molecules may be engaged by CB_1Rs in a manner independent of cAMP and MAP kinases (13). Knocking down β-arrestin1 selectively abolished the cannabinoid sensitivity of leptin signalling whereas silencing β-arrestin2 had no such effect. Moreover, CB_1Rs also lost their influence

on STAT3 activity in a HEK cell line lacking β-arrestin2 thus corroborating the notion that β-arrestin1 is required for the cannabinoid mediated inhibition of leptin signalling.

The question then arose as to how β-arrestin1 conveys the CB_1R mediated inhibition on STAT3, that is, which molecule is the direct effector of CB_1Rs. To address this problem, we systematically targeted protein tyrosine phosphatases first with broad range, then with more selective pharmacological inhibitors. The screening identified the nuclear phosphatase PTPN2 (T-cell protein tyrosine phosphatase, TCPTP, also referred to as TC45) as an indispensable component of the CB_1R– STAT3 axis. Indeed, selective knock-down of TCPTP fully abolished the CB_1R effect on leptin signalling while silencing the related phosphatase PTP1B was ineffective in this respect.

At this point, the connection between β-arrestin1 and TCPTP was still lacking. Analysis of the cellular distribution of β-arrestin1 with our automated confocal microscopic approach revealed that simultaneous activation of CB_1 and leptin receptors induces partial translocation of β-arrestin1 from the cytosol to the nucleus. This observation hinted at a possible direct molecular interaction between β-arrestin1, TCPTP *and* phospho-STAT3, not unlike to that reported for STAT1 (14). Indeed, we were able to demonstrate with co-immunoprecipitation experiments the direct binding of both TCPTP *and* phospho-STAT3 to β-arrestin1 in CB_1R agonist and leptin co-stimulated neurons. Thus, β-arrestin1 acts as a quasi-scaffold protein enabling effective STAT3 dephosphorylation by TCPTP (Fig.1.).

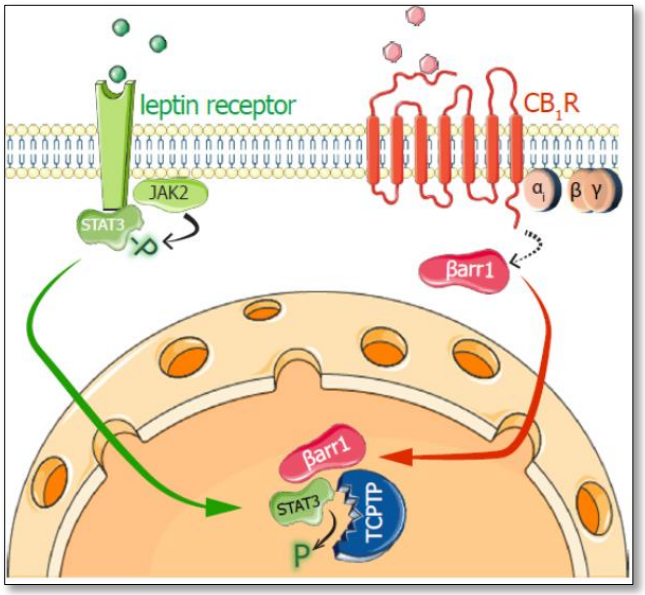

Figure 1. Our proposed model of CB_1R mediated inhibition of leptin induced signal transduction in neurons

In the light of the above findings, we started breeding β-arrestin1 knock-out mice in order to show that β-arrestin1 is in fact necessary for the CB_1R-dependent inhibition of leptin signalling in *in situ* neurons. The data from the first cohort of animals seem to corroborate our model (Fig.1.); the second set of knock-out mice, necessary to reach the desired number of observations, will be ready for experimentation shortly. All other data are prepared for submission. We are confident that our study is a relevant step towards deciphering the early steps of leptin resistance and weight gain and, therefore, will firmly aim at a high-impact (D1) journal in the field of metabolism when publishing our results.

Further studies

During the course of the above study, we developed a number of recombinant proteins that proved to be valuable assets in other experiments as well. For instance, we cloned wild-type and enzymatically inactive mitochondria-targeted soluble adenylyl cyclase isoforms and discovered a novel intramitochondrial Ca^{2+} – cAMP interaction pathway that boosts steroid synthesis. We also identified a soluble adenylyl cyclase isoform unique to the human adrenal cortex (12).

We recognized early on that recombinant CB_1Rs expressed with conventional vectors tend to suffer from overt proteasomal degradation, non-canonical coupling to G_s proteins and atypical cellular localization. In order to overcome these issues, we tailored the promoter region of the vectors to yield CB_1R expression levels close to that observed for the endogenous receptors. The resulting constructs mimicked the biological behaviour of endogenous CB_1 receptors considerably well and were virtually free of proteasomal degradation and non-canonical signalling events (15).

Using our improved CB_1R constructs, we demonstrated in collaboration that hepatocyte CB_1Rs alter the soluble leptin receptor concentration of the plasma and thereby contribute to leptin resistance (16). We also found that, in renal proximal tubule cells, CB_1R activation by hyperglycaemia drives GLUT2 overexpression and ensuing diabetic nephropathy (Hinden *et al.*, Nature Communications, under revision).

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