Melanocortin receptors in leptin effects

Leptin acts on the central nervous system to cause a reduction in food intake and body weight[1,2]. The melanocortin system in the brain is also implicated in energy homeostasis, with agonists of the melanocortin-4 (MC4) receptor reducing food intake[3] and targeted mutation of the MC4 receptor causing obesity[4]. We now show that MC4 receptor signalling is an important mediator of leptin's effects on food intake and body weight, demonstrating a link between the two systems.

Leptin receptors are on proopiomelanocortin (POMC) neurons in the arcuate nucleus[5] that synthesize melanocortins, therefore it seemed likely that leptin-induced reductions in food intake might be mediated by the melanocortin system. To test this hypothesis, we determined whether pharmacological blockade of MC4 receptors attenuates the ability of leptin to reduce food intake or body weight. Because leptin also activates the hypothalamic paraventricular nucleus (PVN), as measured by c-Fos expression, we also wished to determine whether this response involves melanocortin receptors.

We injected male Long–Evans rats with the non-selective MC4 receptor antagonist SHU9119 into the third cerebral ventricle (i3vt). There was a significant increase in food intake 4 and 24 h after administration of 1 nmol SHU9119, whereas 0.5 nmol altered neither food intake nor body weight at any time point. We therefore pretreated rats with 0.5 nmol of SHU9119 or vehicle i3vt before an i3vt injection of 3.5 μg of recombinant leptin or its vehicle (see ref. 1 for details). Consistent with previous findings[1], leptin alone significantly reduced food intake after 4 h (−54%) and after 24 h (−46%, $P<0.05$; Fig. 1a), and reduced body weight after 24 h (−18.6 g, $P<0.05$). However, when these same animals were pretreated with SHU9119, leptin did not affect food intake or body weight.

To assess the specificity of SHU9119 to reverse anorexia induced by leptin, we administered glucagon-like-peptide-1 (7–36) amide (GLP-1), a potent inducer of anorexia when given intracerebroventricularly[6–9]. The dose of GLP-1 (10 μg i3vt) that we selected reduces short-term food intake to an extent comparable to 3.5 μg leptin[7] (−63% after 4 h) and this effect was not altered by pretreatment with 0.5 nmol SHU9119 (Fig. 1b).

We assessed cFos-like-immunoreactivity in the PVN after leptin treatment with or without SHU9119 pretreatment (see ref. 10 for methods). Consistent with our previous findings[10], leptin increased c-Fos-like immunoreactivity in the PVN by 254%, and this was completely blocked by SHU9119 (Fig. 1c).

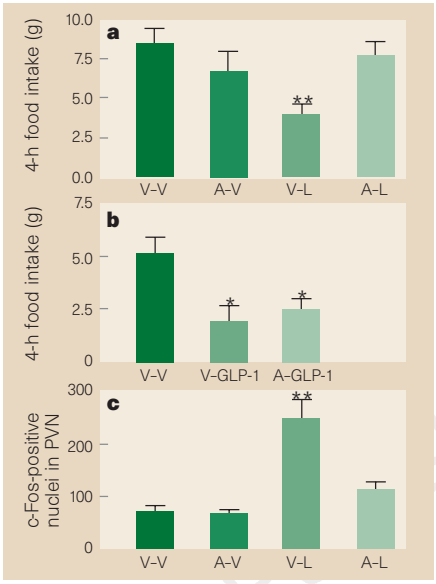

Figure 1 Effect of treatment with a melanocortin receptor antagonist on feeding and the leptin response (means±s.e.m.). V, vehicle; A, antagonist; L, leptin. Data were analysed by a one-way ANOVA followed by post-hoc Neuman-Keul's tests. * Significant difference from the vehicle-only group at $P<0.05$; ** at $P<0.01$. **a,** Effect of pretreatment with i3vt SHU9119 (0.5 nmol) on the anorexic response to i3vt leptin (3.5 μg) on 4-h food intake. Leptin treatments, $n=12$; vehicle treatments, $n=6$. **b,** The effect of pretreatment with i3vt SHU9119 (0.5 nmol) on the anorexic response to i3vt GLP-1 (10 μg) on 4-h food intake. GLP-1, $n=5$; vehicle group, $n=4$. **c,** Effect of pretreatment with i3vt SHU9119 (0.5 nmol) on c-Fos activation in the PVN of the hypothalamus produced by i3vt leptin (3.5 μg) treatment. Vehicle-only, $n=5$; antagonist-vehicle, $n=4$; leptin-treated groups, $n=6$.

Our findings provide direct evidence that MC4 receptor signalling is important in mediating leptin's effect on food intake and body weight. We suggest that obesity stemming from disrupted MC4 receptor signalling (for example, MC4-R knockout and *agouti* mice) results from the loss of an important downstream target in the leptin signalling cascade. The possibility that the melanocortin system is one mediator of leptin's action in the central nervous system is particularly intriguing in light of the recent finding that human obesity and hyperleptinaemia are strongly linked to a region of chromosome 2 near the POMC gene locus[11]. The hypothesis that polymorphisms of the POMC gene are associated with leptin resistance and obesity is consistent with our findings implicating melanocortins in the leptin signalling pathway, and may have important implications for the pathogenesis and treatment of human obesity.

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1. Seeley, R. J. *et al. Hormone Metabol. Res.* **28,** 664–668 (1996).
2. Campfield, L. A., Smith, F. J., Gulsez, Y., Devos, R. & Burn, P. *Science* **269,** 546–549 (1995).
3. Fan, W., Boston, B. A., Kesterson, R. A., Hruby, V. J. & Cone, R. D. *Nature* **385,** 165–168 (1997).
4. Huszar, D. *et al. Cell* **88,** 131–141 (1997).
5. Cheung, C. C., Clifton, D. K. & Steiner, R. A. *Endocrinology* **138,** 4489–4492 (1997).
6. Tang-Christensen, M. *et al. Am. J. Physiol.* **271,** R848–R856 (1996).
7. Thiele, T. E. *et al. Am. J. Physiol.* **272,** R726–R730 (1997).
8. van Dijk, G., Thiele, T. E., Seeley, R. J., Woods, S. C. & Bernstein, I. L. *Nature* **385,** 214 (1997).
9. Turton, M. D. *et al. Nature* **379,** 69–72 (1996).
10. van Dijk, G. *et al. Am. J. Physiol.* **271,** R1096–R1100 (1996).
11. Comuzzie, A. G. *et al. Nature Genet.* **15,** 273–276 (1997).

Transcriptional squelching re-examined

The introduction of a potent transcriptional activator into eukaryotic cells can paradoxically suppress the transcription of a co-introduced target gene[1–3]. This so-called 'squelching' is thought to result from titration of one or more general transcription factors (GTFs)[4], indicating that GTFs might be in limited supply within the cell[5,6]. We now find that in mammalian cells, squelching is limited to episomal target genes, whereas genes integrated into cellular chromatin are immune.

During the process of designing more potent transcriptional activation domains for use in gene therapy, we constructed chimaeric transcription factors composed of the yeast GAL4 DNA-binding domain and activation domains derived from the herpes simplex virus protein VP16[7] and the NF-κB p65 protein[8]. We tested the proteins on a target gene composed of a secreted alkaline phosphatase (SEAP) reporter under the control of a minimal human interleukin-2 (IL-2) gene promoter flanked by five GAL4 binding sites.

When we transiently introduced the

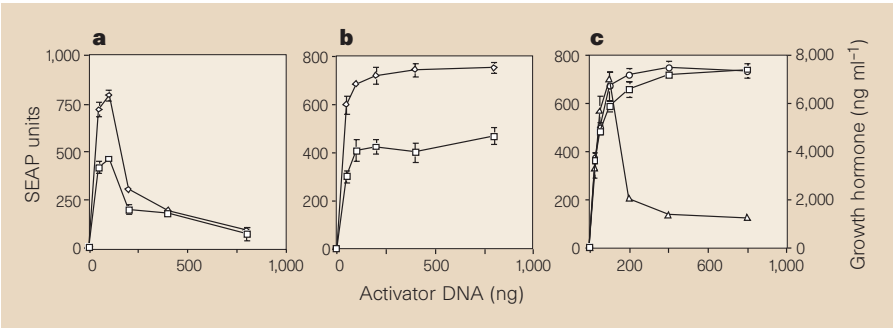

Figure 1 Transcriptional activation of genes embedded in chromatin and episomes. Increasing amounts of plasmids encoding chimaeric activator proteins GAL4-p65 (diamonds) and GAL4-VP16 (squares) were transiently transfected into either **a,** HT1080 cells cotransfected with reporter plasmid 5xGAL4-IL2-SEAP or **b,** HT1080B cells carrying the stably integrated reporter gene pLH-5xGAL4-IL2-SEAP. **c,** Increasing amounts of GAL4-p65 expression vector were transiently transfected into HT1080B cells in the presence or absence of co-transfected hGH reporter plasmid (5xGAL4-IL2-hGH). We measured the transcriptional activity of the integrated SEAP gene in the presence (squares) or absence (circles) of cotransfected hGH reporter plasmid, as well as the transcriptional activity of the transiently transfected hGH reporter plasmid (triangles). Mean values of SEAP activity and hGH protein secreted into the medium are shown (± s.d.).

reporter gene and expression plasmids encoding the transcription factors into HT1080 human fibrosarcoma cells, we observed peak expression of reporter genes at relatively low levels of input activator plasmid, whereas higher levels of activator led to a sharp reduction in reporter gene expression (Fig. 1a). Such squelching in the presence of high levels of activator has been widely observed[9,10].

We tested the same transcription factors in an HT1080-derived cell line in which the same SEAP reporter gene had been stably introduced by retroviral transduction. Expression of the integrated reporter gene was not inhibited by levels of activator that produced pronounced squelching of a transiently transfected reporter (Fig. 1b). High intracellular concentrations of potent transcriptional activators cannot therefore be titrating GTFs required for transcription of an integrated chromatin-embedded gene.

It is possible that only DNA-bound activators effectively titrate GTFs. If so, the presence of high-copy episomal activator binding sites would be required for squelching to occur. We constructed a second reporter gene in which the same GAL4-driven IL-2 promoter was fused to a human growth hormone (hGH) reporter gene. This plasmid was cotransfected with the GAL4-p65 expression plasmid into cells containing an integrated SEAP reporter gene, thus allowing both reporter genes to be assayed in the same cell population. If, in the presence of a high-copy episomal template, GAL4-p65 titrates the GTFs necessary for activity of this promoter regardless of location, we would expect to see inhibition of both the episomal hGH gene and the integrated SEAP gene. Instead, although expression of the episomal hGH gene was inhibited at high activator concentrations, the integrated SEAP gene responded identically whether the episomal gene was present or not (Fig. 1c). Thus, if the transcriptional activators are indeed titrating one or more GTFs required for transcription of the episomal gene, these GTFs are not required (or are not rate-limiting) for transcription of the integrated chromatin-embedded gene.

There are several possible explanations for our observations. Episomal and integrated genes may have different GTF requirements or rate-limiting steps for transcription. Limiting GTFs may bind with higher affinity to a chromatinized promoter, allowing chromatinized promoters to compete more effectively for GTFs. Episomal and integrated genes may reside in different compartments of the nucleus with independent GTF pools, or the conformational flexibility of an episomal DNA molecule may permit the formation of nonproductive transcription factor complexes that cannot form in the more constrained environment of chromatin. Whatever the explanation, the idea that GTFs are limiting for gene transcription in mammalian cells, an idea based largely on transient transfection assays, needs to be reconsidered.

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1. Gill, G. & Ptashne, M. *Nature* **334,** 721–724 (1988).
2. Berger, S. L. *et al. Cell* **61,** 1199–1208 (1990).
3. Berger, S. L. *et al. Cell* **70,** 251–265 (1992).
4. Orphanides, G., Lagrange, T. & Reinberg, D. *Genes Dev.* **10,** 2657–2683 (1996).
5. Ptashne, M. & Gann, A. A. *Nature* **346,** 329–331 (1990).
6. Guarente, L. *Trends Biochem. Sci.* **20,** 517–521 (1995).
7. Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. *Nature* **335,** 563–534 (1988).
8. Schmitz, M. L. & Baeuerle, P. A. *EMBO J.* **10,** 3805–3817 (1991).
9. Zhu, H., Joliot, V. & Prywes, R. *J. Biol. Chem.* **269,** 3489–3497 (1994).
10. Liu, X. & Berk, A. J. *Mol. Cell. Biol.* **15,** 6474–6478 (1995).

Immunosuppressive effects of apoptotic cells

Apoptotic cell death is important in the development and homeostasis of multicellular organisms[1] and is a highly controlled means of eliminating dangerous, damaged or unnecessary cells without causing an inflammatory response or tissue damage[1,2]. We now show that the presence of apoptotic cells during monocyte activation increases their secretion of the anti-inflammatory and immunoregulatory cytokine interleukin 10 (IL-10) and decreases secretion of the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α), IL-1 and IL-12. This may inhibit inflammation and contribute to impaired cell-mediated immunity in conditions associated with increased apoptosis, such as viral infections, pregnancy, cancer and exposure to radiation.

The lack of an inflammatory response during apoptosis has been attributed to the rapid phagocytosis of apoptotic cells before cell lysis, thereby preventing the release of noxious contents which could provoke inflammation and tissue damage[2]. However, potent inducers of apoptotic cell death such as ultraviolet irradiation and X-rays ameliorate inflammatory diseases[3,4]. We therefore investigated whether apoptotic cells simply fail to provide pro-inflammatory signals or whether they also actively suppress an inflammatory response.

Bacterial lipopolysaccharides (LPS) are potent stimulators of cytokine secretion from monocytes and macrophages. We tested whether apoptotic cells modify the LPS-induced cytokine secretion pattern of peripheral blood mononuclear cells (PBMC) and purified monocytes. In the presence of apoptotic peripheral blood lymphocytes (PBL), PBMC and monocytes produced substantially more of the anti-inflammatory cytokine IL-10 and less of the pro-inflammatory cytokines TNF-α, IL-1β and IL-12 (Fig. 1). In contrast, living or freshly prepared paraformaldehyde-fixed PBL did not alter the cytokine secretion pattern (Fig. 1a,b and data not shown).

Various human and murine cells irradiated with ultraviolet light or γ-rays or deprived of growth factor had a similar anti-inflammatory effect on human monocytes, indicating that the anti-inflammatory properties of apoptotic cells are conserved between mammalian species and are independent of the apoptosis-inducing stimulus. The incubation time between monocytes and apoptotic cells before LPS stimulation influences the changes in the cytokine secretion pattern. Whereas the induction of IL-10 is highest without pre-incubation, the relative inhibition of pro-