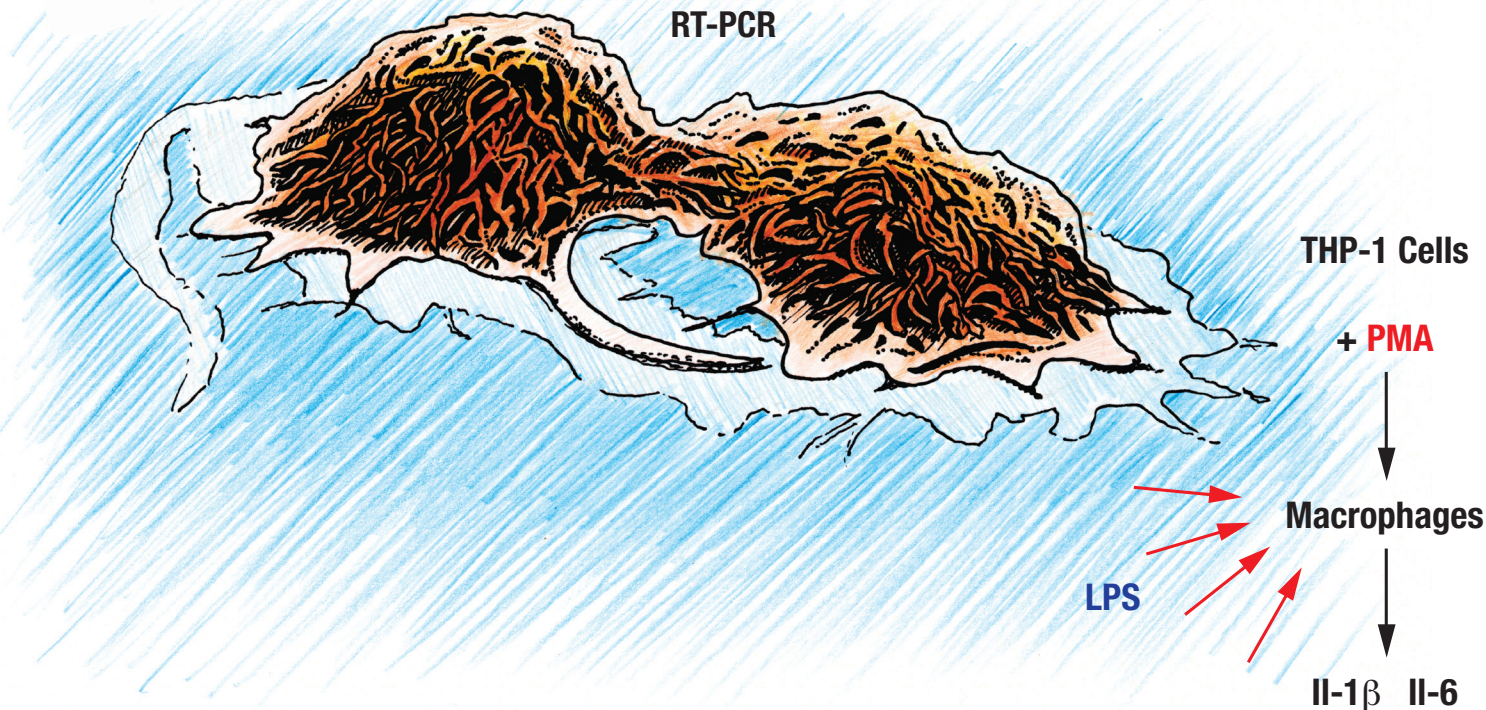
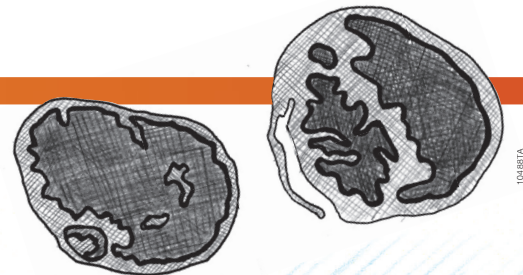


Lysosomal Pathways and Glucocorticoid Receptor Signaling

Review of: Yuanxheng He *et al.* 2011 *Sci. Signal.* 4 (180) ra44



RT-PCR



OBSERVATIONS

- Chloroquine (CQ) accumulates in lysosomes
- CQ ↑ pH in lysosome; impairs function
- Lysosome important for antigen processing and presentation
- CQ ↓ levels of circulating cytokines (IL-1β & IL-6)
- IL-1β & IL-6 repressed by glucocorticoid receptor (GR) signaling

Stimulate cells with LPS under a variety of conditions and look at abundance of IL-1β and IL-6 transcripts (repressed by GR signaling)

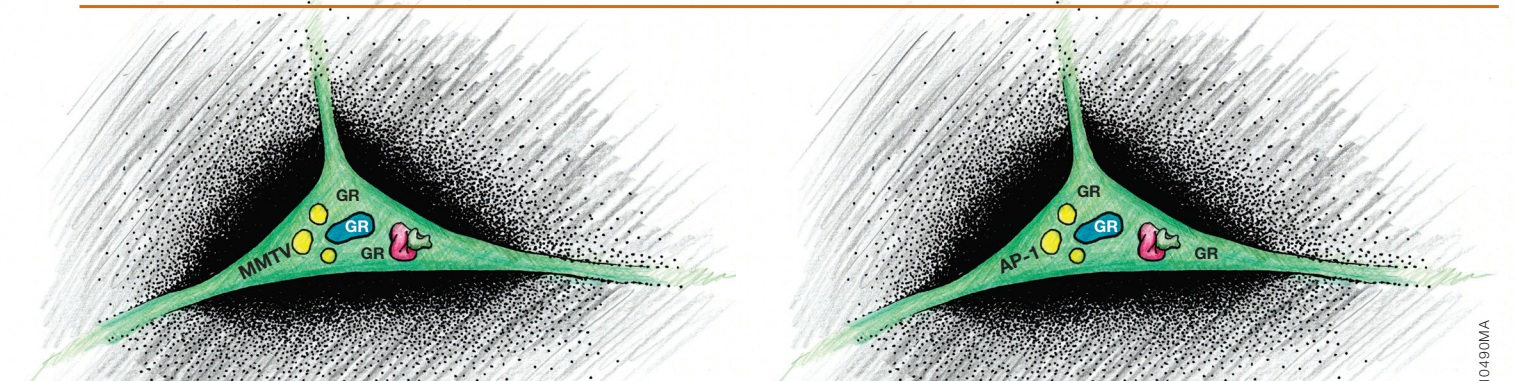
- + CQ: ↓ IL-1β and IL-6 mRNA
- + CQ and Dexamethasone: ↓↓ mRNA levels versus Dexamethasone alone
- + Lysosomal V-ATPase inhibitors: ↓↓ mRNA levels
- + Knockdown TFEB (required for lysosome biosynthesis): ↓↓ mRNA levels

Expression Profiling THP-1 Cells, LPS-Stimulated

Identified genes in which CQ and Dexamethasone exerted synergistic effect. Synergistically represses genes included:

- Phospholipase 2A => prostaglandin-mediated inflammation
- Several chemokines => activators of inflammation

Dual Reporter Assays

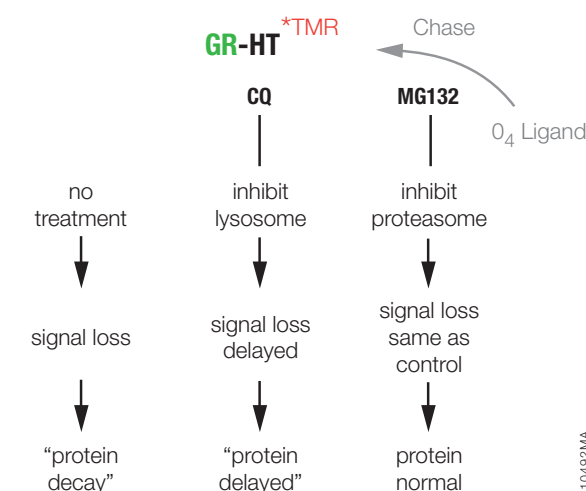


AD293 Cells Expressing GR and MMTV Luc Activation or AP-1 Luc Repression

- CQ ↑ MMTV-Luc ↓ AP-1 Luc
- CQ enhances dexamethasone effect on GR signaling
- “stripped” serum – lose the effects
- Knock out TFEB: ↑ MMTV-luc
- Over express TFEB: lose GR-mediated regulation
- Inhibit V-ATPase: enhance GR-mediated regulation
- Similar effects on reporters seen for estrogen & Androgen receptors

HaloTag® Labeling

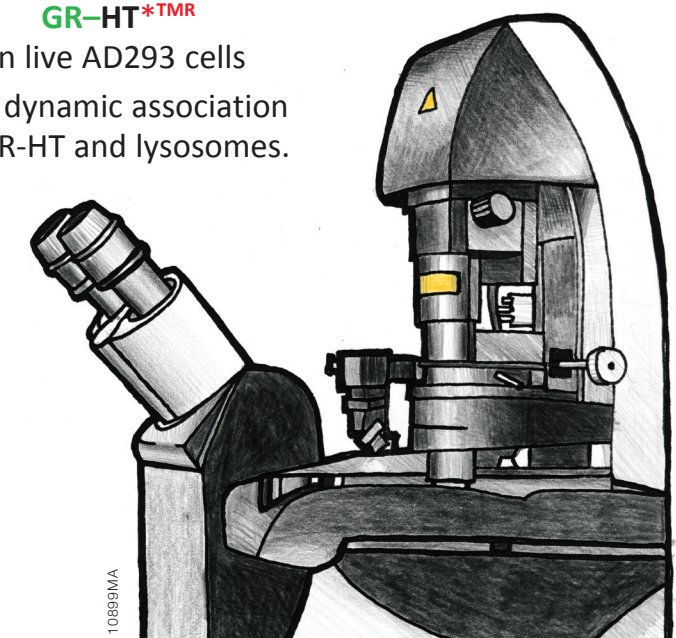
Pulse-Chase Experiments



GR-HaloTag® Fusion

Live-Cell experiments

Follow GR-HT^{*TMR} in live AD293 cells
Saw dynamic association of GR-HT and lysosomes.



In AD293 cells lysosome is responsible for stability of GR.

Chloroquine, Glucocorticoids and Inflammation: A Case Study

Review of He, Y. et al. (2011) Identification of a Lysosomal Pathway that Modulates Glucocorticoid Signaling and the Inflammatory Response. Sci. Signal. 4(180), ra44.



Introduction

In a recent article published in *Science Signaling*, Yuangheng He and colleagues asked how chloroquine (CQ) enhances the anti-inflammatory effects of synthetic glucocorticoids like dexamethasone, which are used to treat a host of inflammatory and autoimmune diseases. In the process they explored the intersection of lysosomal degradation pathways and glucocorticoid receptor signaling. For these investigations, they needed tools such as reporters and protein tags that allowed sensitive an accurate detection of events in real time in a variety of cells and systems.

Here we will summarize the experiments presented in this paper.

Observations and Prior Knowledge

- 1. CQ accumulates in the lysosome.
- 2. CQ raises the pH in the lysosome and hinders lysosome function.
- 3. The lysosome is important for antigen processing and presentation, and therefore plays a role in immune response and inflammation.
- 4. There is a decrease in circulating cytokines (like IL-1β and IL-6) in individuals treated with CQ.
- 5. These same cytokines are repressed by glucocorticoid signaling.

Is the effect of CQ on cytokine levels occurring at the protein or the mRNA level?

The authors used RT-PCR to determine whether CQ treatment affected the abundance of mRNA for IL-1β and IL-6 in lipopolysaccharide (LPS)-stimulated THP-1 cells. THP-1 cells can be induced to differentiate into macrophages by treatment with the phorbol ester, PMA.

They observed that CQ dramatically reduced the abundance of these transcripts in a manner similar to the glucocorticoid, dexamethasone.

Does CQ modulate activity of reporters under the control of GR elements in a manner similar to dexamethasone?

AD293 cells coexpressing the glucocorticoid receptor (GR) and luciferase reporter gene under the control of either MMTV (MMTV-*luc*) or AP-1 (AP-1-*luc*) were exposed to CQ, and reporter responses measured. MMTV contains glucocorticoid response elements (GRE) and is activated by GR. The reporter under the control of AP-1 is repressed by GR, which interacts with the transcription factor AP-1 to repress transcription.

The data indicated that CQ activated MMTV-*luc* and repressed AP-1-*luc* in a dose-dependent manner.

What is the mechanism by which CQ affects GR signaling?

The authors next investigated whether GR distribution, ligand binding or possibly receptor/chaperone interaction was influenced by CQ. Their investigations revealed a modest increase in maximal binding of GR to ligand.

Previous reports indicated that CQ increased GR signaling activity in rat liver extract, which the authors felt was consistent with their observations that CQ increased GR-dexamethasone binding activity. With this information in mind, they looked at whether CQ enhanced GR signaling in the presence of a glucocorticoid such as dexamethasone.

Does CQ enhance the effect of the glucocorticoid, dexamethasone, on GR activation and repression?

The authors returned to the luciferase reporter constructs and the AD293 cells. AD293 cells expressing the GR and either MMTV-*luc* or AP-1-*luc* were treated with both dexamethasone and CQ or dexamethasone alone. The dual-reporter assay results showed that CQ enhanced both GR-mediated activation and GR-mediated repression of the reporter genes, and this was observed with all concentrations of dexamethasone tested. The same effect was also observed with four other synthetic glucocorticoids and the endogenous hormone cortisol.

Does CQ affect the signaling of endogenous GR ligands in serum?

The authors “stripped” serum of endogenous hydrophobic steroids by treating it with charcoal. AD293 cells expressing the GR and either MMTV-*luc* and AP-1-*luc* were treated with CQ in the presence of “stripped” or normal serum. Reporter assays showed that CQ had less of an effect on reporter activity in the stripped

serum. Furthermore in LPS-stimulated THP-1 cells treated with CQ in the presence of stripped or normal serum, CQ did not repress IL-1β or IL-6 mRNA transcription in stripped serum to the extent that it did in normal serum. Similar results were observed with mouse macrophage cells.

What genes, targeted by GR signaling, are synergistically affected by CQ?

To gain a more complete picture of the genes that might be affected by CQ stimulation of GR signaling, the authors performed gene expression profiling in LPS-stimulated THP-1 cells to identify genes in which CQ and dexamethasone exhibited a synergistic effect. Synergy was defined as the effect of treatment with dexamethasone and CQ resulting in a response (either repression or activation) that was more than the response generated by dexamethasone alone plus that generated by CQ alone. In their analysis of genes that were synergistically repressed in the presence of CQ and dexamethasone, they identified the gene encoding phospholipase A2, a protein that is involved in prostaglandin-mediated inflammation. They also identified several chemokines, many of which are associated with inflammation in autoimmune diseases. Additionally, five of these synergistically repressed chemokines are critical for monocyte- and macrophage-induced inflammation. These results suggest that GR signaling represses major inflammatory chemokines induced by macrophage cells and that CQ enhances this effect.

So where does the lysosome fit into this story? Will other compounds that inhibit lysosomal function have similar effects on GR signaling?

Chloroquine (CQ) is a weak alkaline compound that accumulates in lysosome, raising the pH of this organelle and interfering with lysosomal function. The authors sought to determine if there was any relationship between lysosome function and GR signaling. Bafilomycin A and concanamycin A are two inhibitors of the V-ATPase proton pump in the lysosome. This proton pump is essential for maintaining the acidic environment of the lysosome, and inhibiting it interferes with the normal lysosomal functions of degrading and recycling cellular components. The authors again turned to the Dual-Glo® Assay to ask if AD293 cells expressing GR and MMTV-*luc* show enhanced dexamethasone signaling when treated with bafilomycin A1 or concanamycin A. The same compounds showed enhanced repression of IL-1β and IL-6 mRNA in LPS-stimulated THP-1 cells. Furthermore, when siRNAs were used to knock down individual peptide components of the V-ATPase proton pump in AD293 cells, the authors also observed increased GR activation of the MMTV-*luc* reporter, indicating that interfering with lysosomal function enhances glucocorticoid signaling.

TFEB is the master transcriptional regulator of lysosomal biogenesis, regulating the number of lysosomes in response to a variety of cellular signals and stresses. The transcriptional activity of TFEB is correlated with the number of lysosomes. The authors asked: if TFEB signaling is knocked down, will GR signaling be enhanced?

They showed that knockdown of TFEB expression increased dexamethasone-induced gene expression, and in LPS-stimulated THP-1 cells, knockdown of TFEB expression mimicked the effect of CQ on IL-1β and IL-6 mRNA abundance. Conversely, overexpression of TFEB decreased the gene expression of the MMTV-*luc* reporter in the presence or absence of dexamethasone, indicating that TFEB overexpression, and presumably overabundance of lysosome function, interfered with GR signaling.

Is the signaling of other steroid hormone receptors also modulated by CQ and lysosomal function?

The authors performed reporter assays in AD293 cells transfected with either the MMTV-*luc* reporter (androgen receptor [AR]) activation or 3xERE-*luc* reporter (estrogen receptor [ER]) activation. They found that CQ increased transcriptional activation of the AR ligand R1881 and that it also increased the transcriptional activity of the ER ligand E2. Additionally, overexpression of TFEB decreased the ligand-mediated reporter expression for both AR and ER.

Is lysosomal degradation responsible for controlling the stability of the cytoplasmic GR?

Since the authors had data indicating that impairing lysosomal function enhances GR-signaling and that increasing the number of lysosomes interferes with GR-signaling, they asked if lysosomal degradation is responsible for controlling GR stability in the cytoplasm.

There are two major sites for protein degradation in the cell: the proteasome and the lysosome. To investigate the roles of each of these in GR stability, the authors performed a pulse-chase experiment using the HaloTag® protein and ligands. They created a HaloTag®-GR fusion protein (Halo-GR), which they labeled with the fluorescent HaloTag® TMR Ligand. This ligand binds covalently to the HaloTag® part of the fusion. They then chased the label with the nonfluorescent HaloTag® Amine (O4) ligand. In the absence of exogenous GR ligand, the fluorescent signal from the TMR-labeled fusion protein decayed, indicating degradation. When they added CQ to inhibit lysosome function (but not proteasome function), the degradation (as indicated by loss of fluorescent signal) was delayed. When they added the proteasomal inhibitor MG132, there was no delay in degradation as indicated by loss of fluorescent signal. Furthermore, knockdown of TFEB also delayed degradation. They concluded that the lysosome is responsible for degradation of cytoplasmic GR in AD293 cells.

When they added a GR ligand, like dexamethasone, MG132 did appear to delay degradation. Presumably this is because the receptor-ligand complexes are localized in the nuclei where there are no lysosomes, and proteasomes would be the major player in degradation. CQ however did enhance GR stability, even in the presence of dexamethasone.

Experiments in other cell lines did indicate that the major pathways of degradation for the GR may well be cell-line specific, and more experiments will be required to fully understand why this process is different in different cells.

The authors also took advantage of the ability to use the HaloTag® technology to track the fusion protein in live cells to follow synthesis, trafficking and degradation. Again in live AD293 cells, CQ delayed degradation.

Summary

The authors paint an incredibly complete picture of the interaction of three cellular processes: glucocorticoid receptor signaling, lysosomal function, and inflammation. To tease out the interactions of this complex story they employ a variety of flexible and sensitive tools and technologies: RT-PCR, allowing measurement of gene expression in real time; sensitive reporter assays, allowing detection of gene response to treatments and environments; gene expression profiling, providing a big picture view of cellular responses; protein tags that enable “snapshots” of protein populations that can be followed to understand their fate; and protein tags that provide a window into events in live cells. Together, these tools enable a systems approach to life science research.

Ordering Information

Product	Size	Cat.#
Dual-Glo® Luciferase Assay System	10mg	E2920
HaloTag® TMR Ligand	30µl	G8251
HaloTag® Amine (O4) Ligand	5mg	P6741

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