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APPLICATION NOTE

FoxO1: A role in non-alcoholic fatty liver disease.

This application note demonstrates the applicability of the HTRF Phospho-FoxO1 (Ser256) and Total FoxO1 assays to study FoxO1 in HepG2 hepatocytes.

Abstract

This application note evaluates the applicability of the HTRF® Phospho-FoxO1 (Ser256) and Total FoxO1 assays to the study of FoxO1 phosphorylation in HepG2 hepatocytes, in response to conditions mimicking a state of fatty liver disease and insulin resistance. Experimental results obtained using the FoxO1 HTRF assay agreed with previously published data in HepG2, thus demonstrating the assay's validity. The HTRF FoxO1 assays are currently the only homogeneous quantitative method of detecting phosphorylated and total Foxo1 proteins in cells.

Introduction

Forkhead box-containing O subfamily-1 (FoxO1) is a key transcription factor of the insulin signaling pathway. In conditions of impaired insulin signaling, a decrease in FoxO1 phosphorylation is followed by its translocation from the cytoplasm to the nucleus, where FoxO1's transcriptional activity regulates glucose production, lipolysis, inhibition of lipogenesis, stress resistance, apoptosis, autophagy and inflammation. Steatosis, low grade inflammation, and oxidative stress are believed to play a role in FoxO1 activation.

For research use only. Not for use in diagnostic procedures.

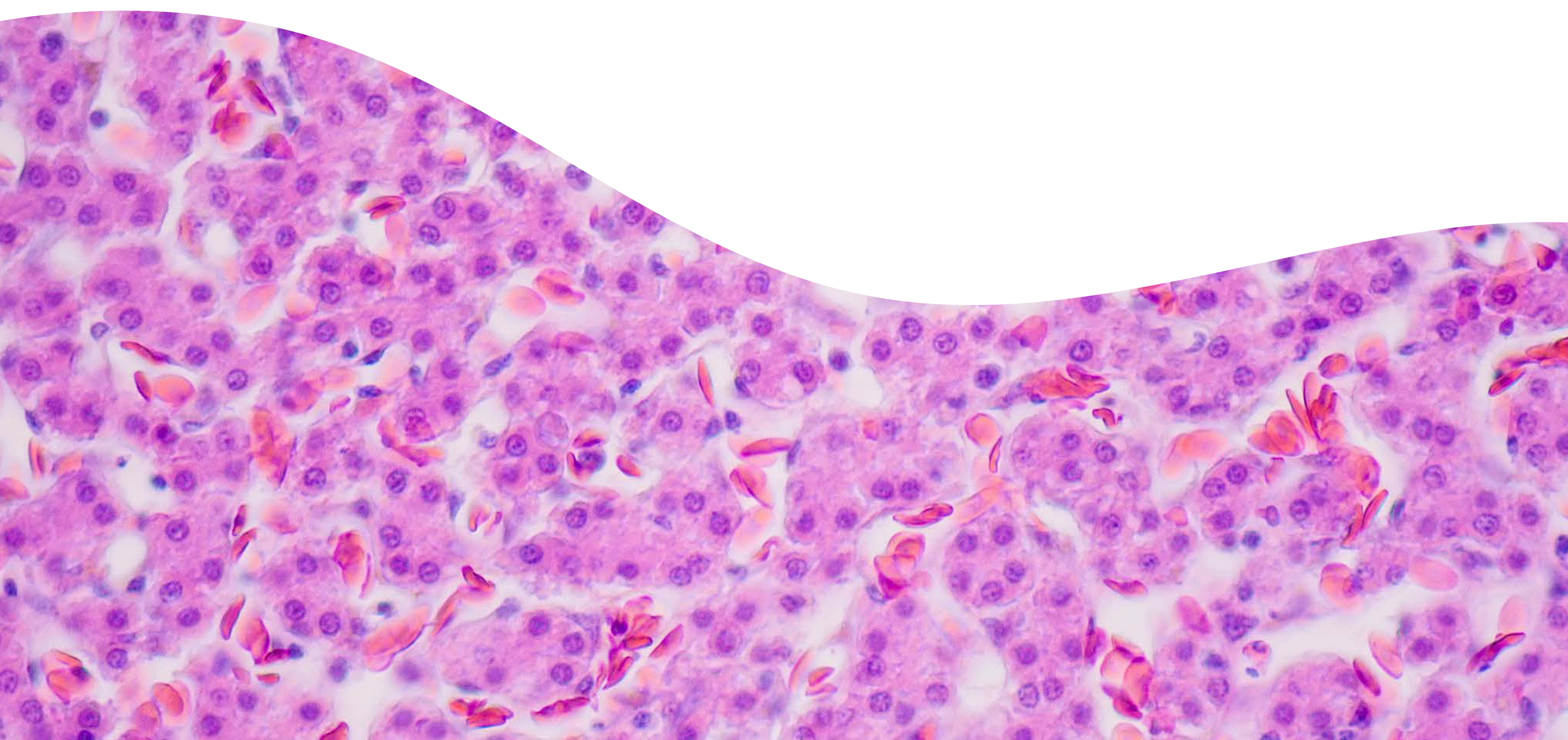


Figure 1: Insulin Signaling Pathway. In impaired insulin signaling, a decrease of FoxO1 phosphorylation is followed by its translocation from the cytoplasm to the nucleus.

Activation of FOXO1 by pro-inflammatory cytokines and oxidative stress

Human hepatoma cell line HepG2 was plated in a 96-well plate (100,000 cells/well) in high glucose culture medium and incubated 24 hours at 37 °C - 5% CO_2. The cells were then treated with a cocktail of TNFα/IL1β/IL6 (30 minutes) or with H_2O_2 (1 hour). After medium removal, the cells were lysed with 50 µL of supplemented lysis buffer #1 for 30 minutes at RT under gentle shaking, and 16 µL of lysate were transferred twice over into a low volume white microplate before adding 4 µL of the HTRF® phospho-FoxO1 or total FoxO1 detection antibodies. The HTRF signal was recorded after 4 hours of incubation.

Results

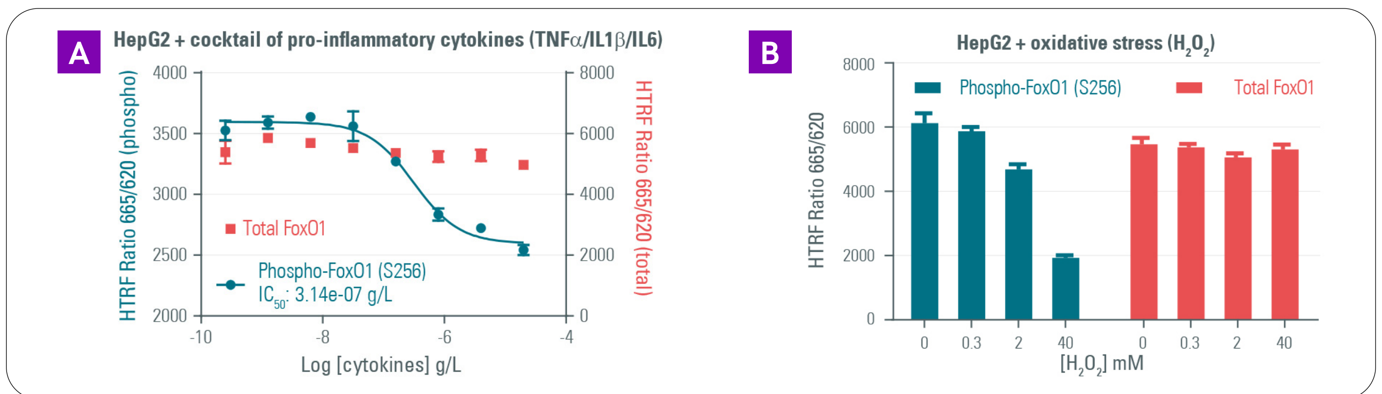


Figure 2: Activation of FoxO1. Treatment of HepG2 cells with pro-inflammatory cytokines (A) and ROS (B) resulted in a decrease in FoxO1 phosphorylation at Serine 256. FoxO1 total protein expression remained constant under both conditions.

Treatment of HepG2 cells with pro-inflammatory cytokines and ROS resulted in a decrease in FoxO1 phosphorylation at Serine 256. FoxO1 total protein expression remained constant under these conditions. Thus, hepatocytes respond to low grade inflammation and oxidative stress by modulating FoxO1 phosphorylation state and this modulation can be reliably detected using the HTRF Phospho-FoxO1 (Ser256) and Total FoxO1 assays.

Comparison of HTRF phospho- and total FOXO1 signaling assay and western blot

Human embryonic kidney cell line HEK293 was seeded in a T175 flask in complete culture medium, and incubated for 2 days at 37 °C, 5% CO_2 until 90% confluency was reached. The cells were then lysed with 3 mL of supplemented lysis buffer #1 for 30 minutes at RT under gentle shaking. Soluble supernatants were collected after a 10-minute centrifugation.

Serial dilutions of the cell lysate were performed in the supplemented lysis buffer and 16 µL of each dilution were transferred into a low volume white microplate before the addition of 4 µL of HTRF phospho- or total detection antibodies. Equal amounts of lysates were used for a side by side comparison between Western Blot and HTRF.

Results

A minimum of 2,000 cells were needed to reliably quantify FoxO1 phosphorylation using the HTRF assay. Compare this with a minimum of 4,000 cells for the Western Blot. The HTRF assay is therefore 2-fold more sensitive than the Western Blot technique. Using the HTRF Total FoxO1 kit, 1,000 cells were sufficient for minimal signal detection while 8,000 cells were needed for a Western Blot signal, demonstrating that the HTRF assay is 8-fold more sensitive than the Western Blot.

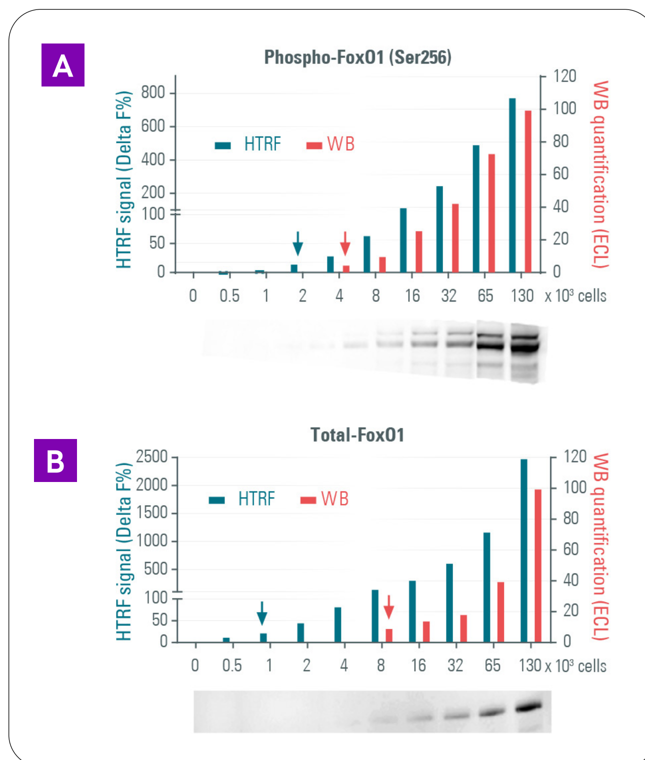
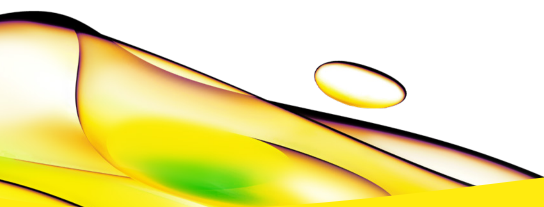


Figure 3: Comparison of HTRF Signaling Assay and Western Blot. A minimum of 2,000 cells were needed to quantify FoxO1 phosphorylation with the HTRF assay compared to 4,000 cells needed for Western Blot (A). For quantifying total FoxO1, 1,000 cells were sufficient for minimal signal detection using HTRF while 8,000 cells were needed for a Western Blot signal (B).

Conclusion

Modulation of FoxO1 phosphorylation could be detected in TNFα/IL1β/IL6-treated HepG2 cells and ROS treated HepG2 using the HTRF Phospho-FoxO1 (Ser256) and Total FoxO1 assays. The results were consistent with those of other investigators, attesting to their physiological relevance and reliability. The HTRF assays displayed superior sensitivity and ease of use over the traditional Western Blot detection techniques.



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