Supporting Online Material for

Evidence That Chromium Modulates Cellular Cholesterol Homeostasis and ABCA1 Functionality Impaired By Hyperinsulinemia

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MATERIALS AND METHODS

Cell Culture. Murine 3T3-L1 preadipocytes were purchased from Dr. Howard Green (Harvard Medical School) and used as previously described [S1]. Briefly, cells were cultured in DMEM containing 25 mM glucose and 10% calf serum at 37^{o}C in an 8% CO_2 atmosphere. Confluent cultures were induced to differentiate into adipocytes as previously described [S2]. All studies were performed on adipocytes which were between 8 and 12 days post-differentiation. Groups supplemented with Cr^{3+} were treated with 1 μM Cr^{3+} picolinate (CrPic) for 16 h as previously described [S2]. Note that numerous control experiments we have performed have consistently shown similar cholesterol lowering action by other forms of chromium (e.g. chloride-bound, CrCl; niacin-bound, CrN) and at pharmacologically relevant doses. Because the use of CrPic best allows for comparisons with our previous studies and the work of others as this is the most commonly used form in such studies, the current study used CrPic, initiated 4 h before chronic exposure to insulin. The AICAR (1 mM) treatment was performed following the 12 h insulin exposure for 45 min, and the DON (20 μM) and βCD (300 μM) treatments were for 12 h during the overnight insulin exposure. Note the low βCD dose used has previously been documented to effectively lower endosomal membrane cholesterol content [S3].

Cholesterol Efflux. ApoA1-mediated cholesterol efflux was determined as described elsewhere [S4]. Briefly, adipocytes were labeled with 0.5 μCi/mL ^{3}H-cholesterol (Sigma Aldrich) for 24 hours in 25 mM glucose DMEM containing 0.2% BSA. Cells were then washed and incubated in the absence or presence of 5 nM insulin to induce insulin resistance (as described above). Cells were then incubated in 25 mM glucose DMEM containing 0.2% BSA and 10 μg/mL lipid-free

ApoA1 for 4 hours. This was followed by measuring ^{3}H-cholesterol in the medium and in the cells. The percentage of acceptor-specific efflux was calculated using the following equation: medium/(medium+cells). Values obtained in the absence of acceptor were subtracted to account for non-specific ^{3}H-cholesterol efflux/leakage.

Subcellular Fractionation and Western Blotting. Plasma membrane (PM), endosomal membrane (EM), and whole cell lysate fractions were isolated as described in [S5]. After addition of 1% NP40 detergent to the prepared fractions, total protein recovered was determined by the Bradford method. Proteins were separated by SDS-PAGE and immunoblotted with either an ABCA1 (Abcam),Rab8 (BD Biosciences), or AMPK (Cell Signaling) antibody, followed by an IRDye™ 700DX or 800DX conjugated secondary (Rockland). Immunoblots were analyzed by Li-COR Odyssey infrared imaging quantification. For subcellular fractions, protein loading was normalized to Ponceau staining and quantified by ImageJ software. For whole cell lysate, blots were normalized to total AMPK or β-actin. Using a portion of the EM fraction, cholesterol was also measured as previously described [S2].

Statistical Analyses. Values are presented as means ±SE. Differences between two groups were analyzed by the Student's *t*-test for independent samples. GraphPad Prism 4 software was used for all analyses. $P < 0.05$ was considered significant.

SUPPLEMENTAL REFERENCES

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