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Exposure to Azide Markedly Decreases the Abundance of mRNAs Encoding Cholesterol Synthetic Enzymes and Inhibits Cholesterol Synthesis

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Abstract This study was performed to identify genes that are regulated in the adaptive response to prolonged inhibition of oxidative phosphorylation. Gene microarray analysis in control Clone 9 cells and Clone 9 cells exposed to 5 mM azide for 24 h was carried out as a condition of ''Chemical hypoxia.'' Among several hundred mRNAs whose abundances were either increased or decreased, we noted that the abundance of mRNAs encoding enzymes that catalyze the sequential steps of cholesterol synthesis was decreased; this finding was verified by real-time PCR. Exposure to azide for 24 h markedly inhibited the biosynthesis of cholesterol by ~90% and decreased the cellular content of cholesterol by 30%, similar results were observed in HepG2 cells. The abundance of sterol regulatory element binding protein (SREBP)-2 mRNA decreased to 0.37 and 0.25 that of controls after 2 and 24 h exposure, respectively. After 24 h of exposure to azide the precursor and nuclear forms of SREBP-2 protein decreased by ~80% and ~50%, respectively. Stimulation of AMP-activated protein kinase (AMPK) by AICAR in Clone 9 cells increased the abundance of mRNAs encoding cholesterol biosynthetic enzymes and that of SREBP-1c, and had no effect on SREBP-2 mRNA abundance. We conclude that the decrease in the abundance of multiple mRNAs encoding cholesterol biosynthetic enzymes may be mediated by decreased expression of SREBP-2 mRNA and protein and does not involve stimulation of AMPK. The decrease in SREBP-2 mRNA and protein abundance in the face of decreased cell cholesterol content raises the possibility of a novel regulatory pathway. J. Cell. Biochem. 100: 1034–1044, 2007.

Key words: cholesterol; gene expression; oxidative phosphorylation; ''chemical hypoxia''; sterol regulatory element binding protein (SREBP); AMP-activated protein kinase (AMPK); mass spectrometry

Mechanisms mediating the adaptation of cells and organisms to states of decreased energy production due to limited supply of oxygen or substrates and/or decreased rates of oxidative phosphorylation are critical to survival and represent an important and active field of inquiry. In previous studies we have noted that exposure of Clone 9 cells to cyanide or azide results in a rapid dose-dependent fall in cell ATP [Mercado et al., 1989; Shetty et al., 1993b], and a rise in ADP and AMP [Jing and Ismail-Beigi, 2006]. Subsequently, cell ATP, ADP, and AMP return to normal levels within an hour and remain at normal levels despite the continuous presence of the inhibitors for more than 24 h [Shetty et al., 1992, 1993b; Jing and Ismail-Beigi, 2006]. The normalization of ATP levels, brought about by an increase in glucose transport and ATP synthesis through anaerobic glycolysis, is associated with an overall decrease in the rate of cellular energy turnover [Mercado et al., 1989; Ismail-Beigi, 1993]. For example, we have noted that global rates of protein [Dufner et al., 2005] and RNA synthesis (unpublished observations) are inhibited by

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~50% in cells exposed to azide for 24 h, while cell ATP levels remain normal. However, synthesis rates of specific RNAs and proteins are stimulated under these conditions [e.g., glucose transporter-1 (Glut1)], presumably as an adaptive response to the prolonged inhibition of oxidative phosphorylation [Shetty et al., 1992, 1993b]. Nevertheless, specific pathways that are either up- or downregulated in the above adaptive response and the mechanisms mediating these changes have not been studied in a systematic fashion.

The present study was performed to better understand pathways that are controlled in response to prolonged energy depletion. Our goal was to identify the set of genes that are positively or negatively regulated in the adaptive response to prolonged inhibition of oxidative phosphorylation. We initiated these studies by performing gene microarray analysis on control Clone 9 cells and Clone 9 cells exposed to 5 mM azide for 24 h as "Chemical hypoxia." Among the 350 mRNAs whose abundances were increased and 400 mRNAs whose abundance were decreased, we noted that the abundance of mRNAs encoding many of enzymes that catalyze the sequential steps of cholesterol synthesis was decreased. These changes were verified by quantitative real-time PCR. We also determined the effect of azide on sterol regulatory element binding protein (SREBP)-2 mRNA and protein abundance in control and azide-treated cells. The rate of cholesterol synthesis was measured by incorporation of deuterium from ^2H_2O into cholesterol, and the effect of azide on cellular cholesterol content was determined. Finally, we examined the potential role of stimulation of AMP-activated protein kinase (AMPK) in the above response. A brief account of our findings has been presented [Kasturi et al., 2005].

MATERIALS AND METHODS

Materials

Cell culture reagents were purchased from Invitrogen (Grand Island, NY). Plastic-ware was from Fisher Scientific. All other chemicals were from Sigma Aldrich or Fisher Scientific, unless otherwise noted. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals Inc. Protein assay kits were purchased from Bio-Rad (Hercules, CA). Cholesterol assay reagents were purchased from Thermo Electron (Louisville, CO). ^2H_2O and GC-MS supplies were purchased from Isotec, Inc. (Miamisburg, OH), and Alltech (Deerfield, IL), respectively.

Cell Culture

Clone 9 cells were maintained in DMEM (5.6 mM glucose) supplemented with 10% bovine calf serum (vol/vol) and 1% penicillin–streptomycin, and were used between passages 25–45. HepG2 cells were maintained in DMEM + F12 (50:50 mixture) with L-glutamine (Cell grow, Media Tech, Inc., Herndon, VA) supplemented with 10% FBS, 1% penicillin–streptomycin and glucose to final concentration of 22.5 mM. CHO-K1 cells were maintained in Ham's F12-Kaighn's modification media (Cell grow, Media Tech, Inc.) supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were transferred to serum-free DMEM, DMEM + F12 or Ham's F12K 24 h before initiation of all experiments. Cells were treated with (final concentrations) azide (5 mM), AICAR (0.1 mM), 25-OH cholesterol (1 μg/ml); all additions were made in a small volume of DMEM.

Microarray Analysis of mRNA Abundance

Total RNA was isolated from control Clone 9 cells and Clone 9 cells treated with 5 mM azide for 24 h. The experiment was performed twice, each in triplicate, and samples were analyzed in parallel from control and azide-treated cells (Affymetrix, Santa Clara, CA). Briefly 5 μg of total RNA (RNeasy, Qiagen, Valencia, CA) was used to synthesize cDNA with a T7-$(dT)_{24}$ primer (Genset, La Jolla, CA) and RT superscript II (Invitrogen) for 1 h at 42°C. After second strand synthesis and removal of contaminating RNA, cRNA was prepared from the cDNA by in vitro transcription with biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY). Biotin-labeled cRNAs (15 μg) were then fragmented by heating at 95°C for 35 min in a buffer containing 40 mM tris acetate pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate.

cRNAs were first hybridized to test arrays to assess the quality of the preparations. Samples were then hybridized to Rat Genome RAE230A microarrays for 16 h at 45°C in a buffer containing 100 mM MES (2-morpholinoethanesulfonic acid) pH 6.6, 1.0 M sodium salts, 0.01% Tween-20, herring sperm DNA (0.1 mg/ml), and

acetylated bovine serum albumin (0.5 mg/ml). Microarray analysis was performed at Gene Expression Array Core Facility of Cancer Center, Case Western Reserve University. The RAE230A microarray represents 31,100 rat gene sequences derived from expressed sequence tag (EST) clusters. Of these sequences, 1/3 are annotated genes (Gen Bank or TIGR). After stringent washing in a microfluidics station, bound cRNA was stained with R-phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) and scanned before and after antibody amplification. Fluorescence intensities were analyzed with a laser confocal scanner (Hewlett Packard). Image output files were inspected for hybridization artifacts, and on the basis of fluorescence intensity differences between perfect-match and -mismatch probes, absolute mRNA detection call (present, marginal, or absent) was determined using a *P* value calculated from a Wilcoxon signed-rank test (Microarray Suite 5.0, Affymetrix). Default parameters optimized by the manufacturer for this microarray chip were used for all detection and change analysis.

Transcript signals, assumed to be proportional to the abundance of mRNA transcripts, were used to determine whether relative transcript levels were altered in azide-treated cells as compared to controls. Scanned images were globally scaled to a target of 1,500 to facilitate the comparison of transcript levels determined using the Wilcoxon signed-rank test to compare individual probe pair data for each transcript between control and azide arrays. From the resultant "*P*" values, change calls (increase, decrease, or no change) were assigned to each transcript. This data was further refined with significance analysis of microarrays (SAM) giving a score to each gene in a gene expression profile on the basis of a change in gene expression relative to the standard deviation of repeated measurements. A relative difference score, d(i), was then assigned relative to the standard deviation of the repeated measurements for each gene. This approach generated a list of 846 gene transcripts out of which 455 were registered as increases and 391 were registered as decreases.

Estimation of mRNA Levels by Real-Time RT-PCR

Total RNA was isolated by RNeasy (Qiagen). cDNA synthesis was carried out using superscript first strand synthesis system for RT-PCR kit (Invitrogen). cDNAs transcribed from equivalent amounts of RNA were used for quantification of mRNAs by real-time RT-PCR using ABI 7000SDS system (Applied biosystems, Foster City, CA). The reaction mixture (total volume of 24 μl contained SYBR® green master mix 12 μl (Applied Biosystems), cDNA (1 μl) and 2 μl of primer mix (1 μl each of forward and reverse primers). Primer design for each gene was by use of Primer Express (Applied Biosystems), and primers were custom-made (Integrated DNA Technologies, Inc., Coralville, IA). Primer sequences of the genes used in real-time PCR are shown in Table I.

Real-time PCR was carried out with denaturation at 94°C for 10 min, followed by 45 two temperature cycles (15 s at 94°C and 1 min at 60°C). A melting curve was recorded at the end of the PCR. Relative quantification of mRNAs was computed using the comparative Ct method, and 16*S rRNA* was used as internal reference [Livak and Schmittgen, 2001]. The relative abundance (fold-change) of an mRNA in azide-treated cells was divided by the mean value of control samples, and the results were averaged. In this calculation, increases register as numbers above 1.0 while decreases register as numbers below 1.0. The variation from the mean in control samples was also calculated.

TABLE I. Primer Sequences Used in Real-Time PCR

Gene symbol[a]	Forward	Reverse
Hmgcs1	GCTTTGGTAGTTGCAGGAGACA	CGTAGGCATGCTGCATGTGT
Hmgcr	TACATCCGTCTCCAGTCCAAAA	CAGGTTTCTTGTCGGTGCAA
Mvk	CAATGGGAAAGTGAGCCTCAA	CGCCACCTTCTTCAGCTTCT
Sqle	AGCTATGGCAGAGCCCAATG	CCCGTCGGCAACAACAGT
Dhcr7	TCACTCCCACCTGCAATGGT	GGATATTCCAGTCCGCTGCTT
SREBP-1c	GGCGGGACCACTGTCACTT	CACGATCCTGACTGCTTGTCA
SREBP-2	TGACCACAATGCCGGTAATG	CCGCCTCTCTCCTTCTTTGG
Glut1	ATCTTCGAGAAGGCAGGTGTGC	GCCAGACCAATGAGATGCAG

[a]Gene names are given in Table II.

The relative abundance of Glut1 mRNA was analyzed as an internal positive control.

Analysis of SREBP-2 Precursor and Nuclear Forms in Membranes and Nuclear Extracts

Total membranes and nuclear extracts were prepared in the presence of a mixture of protease inhibitors including calpain inhibitor using a protocol adapted from Adams et al. [2004]. Protein content was measured using Bio-Rad kit and proteins (50–200 μg) were fractionated by SDS-polyacrylamide gel electrophoresis. Precursor form (membrane fraction; ~121 kDa) and mature form (nuclear extract; ~68 kDa) of SREBP-2 were detected using purified anti-human SREBP-2 (IgG-1D2) [Janowski et al., 2001] and anti-hamster SREBP-2 (IgG-7D4) [Hua et al., 1995] antibodies directed against the N-terminal segment of SREBP-2 (ATCC; Manassas, VA). Antibodies were purified and concentrated using Prosep-A Montage antibody purification kit and concentrator (Millipore Corp. Bedford, MA 01730). These antibodies were used at a dilution of ~1:65 in Western blot analysis. CHO-K1 cells were transfected with pTK-HSV-BP2 (ATCC) using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cell extracts were collected and run on SDS–PAGE as described above, and nitrocellulose membranes were probed for SREBP-2 (IgG-7D4, ATCC) and the HSV tag (monoclonal anti-HSV, Novagen, Madison, WI). Densitometric measurements were done using NIH image/Scion Image (Rel: alpha 4.0.3.2).

Measurement of Cholesterol Synthesis

Clone 9 and HepG2 cells were transferred to their respective serum-free media. ^2H_2O was added at 10% of the total volume of medium in addition with azide (5 mM), AICAR (0.1 mM), or diluent. After treatment periods of 6 or 24 h, a sample of the medium was saved to determine the isotopic enrichment of the ^2H_2O precursor. Cells were washed with cold PBS (2 × 5 ml) and were scraped in PBS using a cell lifter. Samples of cell suspensions were used for measurement of protein content and for analysis of cholesterol synthesis.

^2H-labeling of the media water was determined by exchange with acetone as described by Yang et al. [1998]. Briefly, media water was diluted tenfold with distilled water, 40 μl of diluted sample or standard was reacted with 2 μl of 10 N NaOH and 4 μl of a 5% (v/v) solution of acetone in acetonitrile for 24 h. Acetone was extracted by addition of 600 μl of chloroform followed by addition of 0.5 g Na_2SO_4. Samples were vigorously mixed and a small aliquot of the chloroform was transferred to a GC-MS vial.

Acetone was analyzed using an Agilent 5973N-MSD equipped with an Agilent 6890 GC system; a DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all analyses. The oven temperature program was: 60°C initial, increase by 20°C/min to 100°C, increase by 50°C/min to 220°C and maintain for 1 min. The split ratio was 40:1 with a helium flow of 1 ml/min. The inlet temperature was set at 230°C and the mass spectrometer transfer line was set at 245°C. Acetone eluted at ~1.5 min. The mass spectrometer was operated in the electron impact mode (70 eV). Selective ion monitoring of m/z 58 and 59 was performed using a dwell time of 10 ms/ion.

Analysis of Cholesterol Synthesis

Clone 9 cell suspensions in PBS were hydrolyzed in 1.5 ml of 1 N KOH-ethanol (80% v/v) at 80°C for 3 h. After cooling, 1 ml of water was added and samples were acidified with 12 N HCl. Cholesterol was extracted three times with 4 ml of diethyl ether. Ether extracts were pooled and evaporated to dryness under nitrogen. The dry residues were reacted with 60 μl of bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (Regis, Morton Grove, IL) (TMS) at 60°C for 20 min; this forms the trimethylsilyl derivative of cholesterol. ^2H-labeling of cholesterol was determined using the above-mentioned equipment. The oven temperature was initially held for 1 min at 150°C, then increased by 20°C/min to 310°C and maintained for 8 min. The split ratio was 20:1 with helium flow of 1 ml/min. The inlet temperature was set at 270°C and MS transfer line was set at 310°C. Under these conditions cholesterol elutes at ~11.1 min. Electron impact ionization was used in all analyses. Selected ion monitoring of m/z 368–372 (M0–M4, cholesterol) was performed using a dwell time of 10 ms/ion.

Calculation of Lipid Synthesis

First, the abundance of each ion was corrected for natural enrichment using correction matrix [Fernandez et al., 1996]. Following correction, the fractional contribution of de novo

lipogenesis was calculated using the formula: $[(M1 \times 1) + (M2 \times 2) + (M3 \times 3) + (M4 \times 4)]/(n/^2H\text{-labeling of media water} \times \text{incubation time})$, where "n" represents the number of exchangeable hydrogens, assumed to be 25 for cholesterol [Lee et al., 1994].

Measurement of Cell Count, Protein, and Total Cholesterol

Cell counts were measured using a hemocytometer after trypsinization. Total cholesterol and protein were measured using Bio-Rad kit and Infinity cholesterol liquid stable reagent of Thermo DMA respectively, in samples of cell suspensions prepared by scraping cells in PBS.

Statistical Methods

Results of repeated experiments are presented as mean ± SE. Student's *t*-test or ANOVA were used, as indicated, and a $P < 0.05$ was taken as significant.

RESULTS

Microarray analysis of genes whose mRNA abundances changed >2-fold following a 24-h exposure to 5 mM azide included 350 mRNAs whose abundance increased and 400 that showed a significant decrease. Among the mRNAs showing a decrease in their abundance, we noted that the expression of mRNAs encoding enzymes of the cholesterol synthetic pathway was decreased. Fold-changes along with the symbols of genes encoding the cholesterol synthetic enzymes, and those of insulin-induced gene-1 (Insig-1), sterol response element binding protein-1c (SREBP-1c), and Glut1 are listed in Table II. Values were calculated as binary comparisons, and each value was derived from 18 comparisons. It is apparent that the abundance of mRNAs encoding sequential enzymes involved in cholesterol biosynthesis is downregulated. In addition, the abundance of mRNAs encoding SREBP-1c and Insig-1 were decreased. The microarray chip used in these experiments did not contain probes for SREBP-1a or SREBP-2. The abundance of Glut1 mRNA used as an internal positive control was increased 5.6-fold, as previously described [Shetty et al., 1992, 1993b].

Real-time PCR was used to quantify the relative abundance of some of the mRNAs listed in Table II, and the results are summarized in Table III. The abundance of mRNAs encoding enzymes of cholesterol synthesis was decreased in azide-treated cells after 24 h between 0.41 and 0.03-fold compared to diluent-treated cells. Because of the importance of SREBP-2 as a controller of expression of cholesterol synthetic enzymes, we also measured its abundance by real-time PCR in control and azide-treated cells. The abundance of SREBP-1c and SREBP-2 mRNA decreased to 0.52 and 0.25 of control cells, respectively. Glut1 mRNA

TABLE II. Effect of Azide on Relative mRNA Content of Enzymes Encoding Cholesterol Synthesis Determined by Microarray Analysis

Gene name	Gene symbol	Fold-change ± SE
Cholesterol biosynthesis		
3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1	Hmgcs1	0.15 ± 0.02
3-Hydroxy-3-methylglutaryl-coenzyme A reductase	Hmgcr	0.31 ± 0.08
Mevalonate kinase	Mvk	0.25 ± 0.04
Isopentenyl diphosphate delta isomerase	Idi1	0.14 ± 0.04
Farensyl diphosphate synthase	Fdps	0.38 ± 0.07
Farnesyl diphosphate farnesyl transferase 1	Fdft1	0.47 ± 0.02
Squalene epoxidase	Sqle	0.10 ± 0.04
2,3-Oxidosqualene:lanosterol cyclase	Lss	0.16 ± 0.01
7-Dehydrocholesterol reductase	Dhcr7	0.14 ± 0.01
Insulin-induced gene-1	Insig-1	0.31 ± 0.04
Lipid metabolism related transcription factor		
Sterol regulatory element binding protein-1c	SREBP-1c	0.58 ± 0.03
Glucose transporter		
Glut1	Glut1	5.6 ± 0.3

List of genes encoding enzymes involved in cholesterol synthesis in Clone 9 cells that are modified by treatment with 5 mM azide for 24 h. Fold-changes were calculated from two different experiments each performed in triplicate samples. Values are averages of 18 binary comparisons (mean ± SE). The relative abundance (fold-change) of each mRNA in azide-treated cells was expressed as the ratio of the value in controls. Glut1 mRNA was analyzed as a positive control. All changes listed were significant ($P < 0.05$).

TABLE III. Effect of Azide on Relative mRNA Content of Specific Genes Encoding Cholesterol Synthesis by Real-Time RT-PCR

Gene name	Gene symbol	Fold-change ± SE
Cholesterol biosynthesis		
HMG-CoA synthase	Hmgcs1	0.14 ± 0.02
HMG-CoA reductase	Hmgcr	0.41 ± 0.03
Mevalonate kinase	Mvk	0.14 ± 0.01
Squalene epoxidase	Sqle	0.03 ± 0.01
7-Dehydrocholesterol reductase	Dhcr7	0.05 ± 0.01
Lipid metabolism-related transcription factors		
Sterol regulatory element binding protein-1c	SREBP-1c	0.52 ± 0.05
Sterol regulatory element binding protein-2	SREBP-2	0.25 ± 0.05
Glucose transporter		
Glut1	Glut1	7.00 ± 0.50

Total RNA was isolated from duplicate or triplicate control Clone 9 cells and cells exposed to 5 mM azide for 24 h was assayed by real-time PCR. In each experiment, the relative abundance (fold-change) of mRNA in azide treated cells was expressed as (mean ± SE) fraction of control value except for Glut1. Glut1 mRNA was analyzed as a positive control. The mean and the variation from the mean in control samples was 1.0 ± 0.3. The experiment was repeated 2 to 4 times, and the number of samples for each mRNA analyzed was 6 to 11. All changes listed were significant ($P < 0.05$).

abundance was increased 7.0-fold in azide-treated cells.

In addition we examined the effect of azide on SREBP-2 mRNA abundance after 2 and 24 h of exposure (Fig. 1). Treatment with azide for 2 h decreased the abundance of SREBP-2 mRNA to 0.37 that of controls. Similar to the results shown in Table III, the abundance of SREBP-2 mRNA decreased to ~0.27 that of control cells at 24 h.

We next determined the potential effects of azide on cholesterol synthesis in Clone 9 and HepG2 cells (Fig. 2). The incorporation of deuterium from ^2H_2O into cholesterol was used to determine the percentage of cholesterol synthesized during the 24 h interval in control and azide-treated cells. 25-OH cholesterol, an

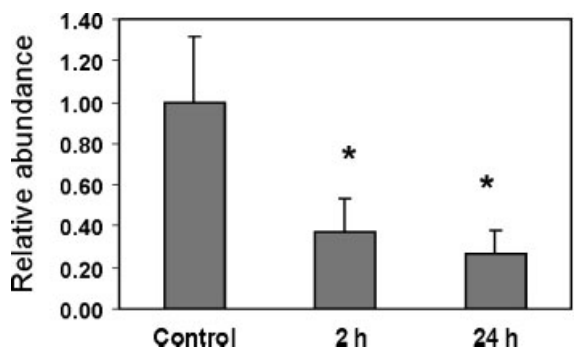

Fig. 1. Effect of azide on the abundance of SREBP-2 mRNA at 2 and 24 h of treatment. Cells were treated with diluent or 5 mM azide for 2 or 24 h in triplicate. Total RNA was isolated and analyzed for the abundance of SREBP-2 mRNA by real-time PCR. Results of two experiments were averaged (mean ± SE; n = 6). * Denotes $P < 0.05$ compared to control cells.

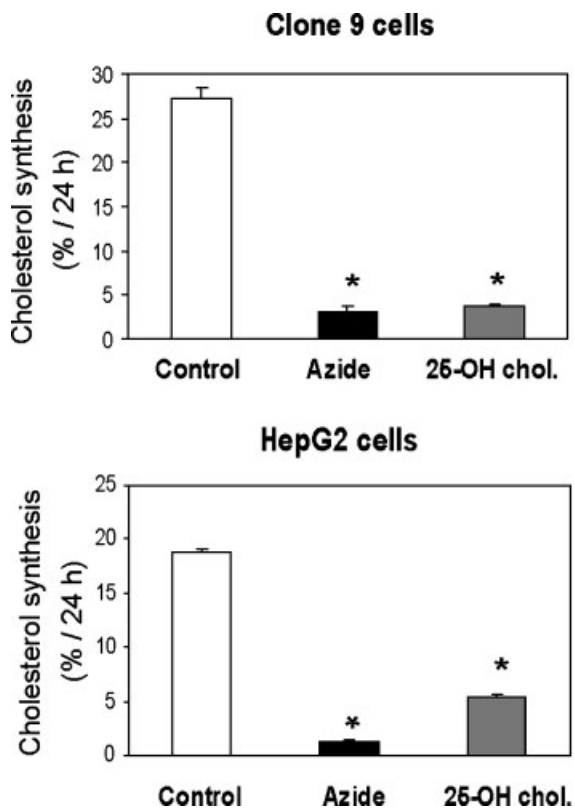

Fig. 2. Effect of azide and 25-OH cholesterol on cholesterol synthesis in Clone 9 cells and HepG2 cells. Clone 9 and HepG2 cells were incubated for 24 h in fresh serum-free DMEM containing 10% ^2H_2O in the presence or absence of diluent, 5 mM azide, or 1 μg/ml 25-OH cholesterol. Values are percent of newly synthesized cholesterol over the 24 h period (mean ± SE; n = 9 to 12). * Denotes $P < 0.05$ versus controls.

agent known to inhibit cholesterol synthesis [Adams et al., 2004] was used as a positive control. Treatment of Clone 9 cells with azide (5 mM) or 25-OH cholesterol (1 μg/ml) for 24 h significantly reduced the percentage of newly synthesized cholesterol from 27.2 ± 1.1 in control cells to 3.2 ± 0.6, 3.7 ± 0.2 in treated cells, respectively. Similar results were obtained in HepG2 cells; the percentages of cholesterol synthesized in 24 h were 18.8 ± 0.3, 1.3 ± 0.2, and 5.5 ± 0.1 in controls, azide, and 25-OH cholesterol-treated HepG2 cells, respectively.

Treatment of Clone 9 cells with azide for 24 h significantly decreased the cholesterol content per dish from 25.1 ± 0.6 to 17.6 ± 1.0 μg (Table IV). Likewise, cholesterol content expressed per unit protein decreased from 27.8 ± 0.9 to 20.0 ± 2.0 μg/mg. These findings are consistent with the measured decrease in cholesterol synthesis during the same time period. Cells exposed to azide exhibited no discernable change in their morphology as examined by microscopy. Treatment of cells with azide resulted in no change in the number of cells per culture dish. Protein content per culture dish decreased by ~9% in cells incubated with azide for 24 h (Table IV).

To determine the mechanism underlying the reduction in cholesterol synthesis and the attendant decrease in cell cholesterol content, we examined whether the decrease in the abundance of SREBP-2 mRNA noted above is associated with decreased abundance of the precursor and nuclear forms of SREBP-2 protein. In accordance with previous reports, the precursor form of SREBP-2 and its nuclear form were identified in Western blots using whole-cell extracts as well as membrane fractions and nuclear extracts [Hua et al., 1993, 1995; Boizard et al., 1998]. In addition, the p- and n-SREBP-2 were verified in CHO-K cells and in CHO cells transiently transfected with N-terminal HSA-tagged SREBP-2 that had been exposed to azide for a similar period of time (data not shown). In repeated experiments employing Clone 9 cells and anti-human or anti-hamster SREBP-2 antibodies, we noted a ~80% and ~50% decrease in the abundance of the precursor and nuclear forms of SREBP-2, respectively, in cells exposed to azide for 24 h (Fig. 3A,B).

TABLE IV. Effect of Azide on Protein, Cell Count, and Cholesterol Content in Clone 9 Cells

	Control	Azide
Protein/dish (μg/dish)	1106 ± 17	1013 ± 34*
Cells/dish $\times 10^6$ cells	7.7 ± 0.2	7.5 ± 0.2
Protein/cells (μg/10^6 cells)	144 ± 6.0	136 ± 4.0
Cholesterol/dish (μg/dish)	25.1 ± 0.6	17.6 ± 1.0*
Cholesterol/protein (μg/mg)[a]	27.8 ± 0.9	20.0 ± 2.0*

Clone 9 cells were treated with diluent or 5 mM azide in triplicate for 24 h and processed to analyze for protein, cell number, and cholesterol content. The experiment was repeated 3 to 4 times. Values are mean ± SE; n = 9 to 12.
*Denotes $P < 0.05$.
[a]These values are from a different set of experiments.

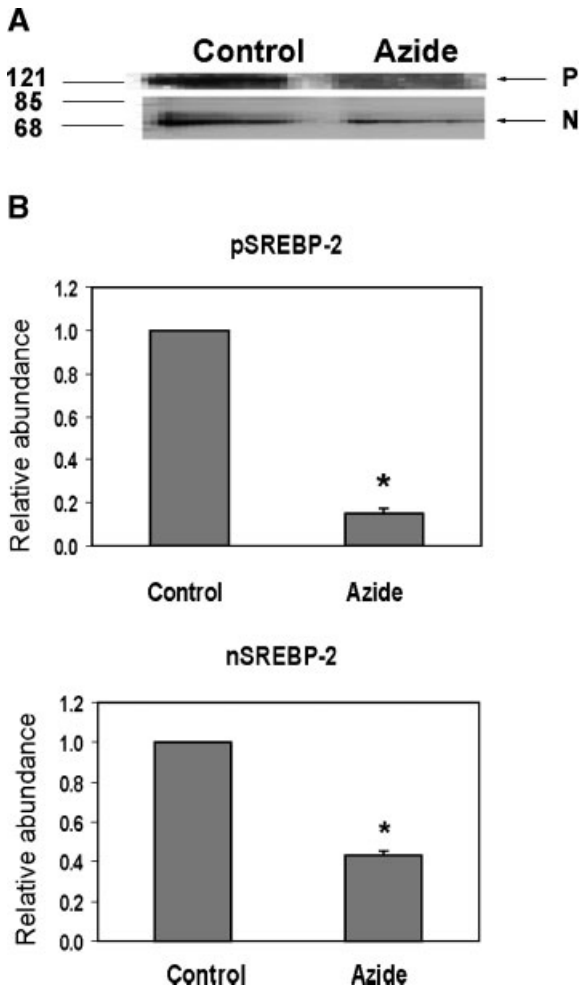

Fig. 3. Western blot of SREBP-2 protein in control and azide-treated cells. **A**: Clone 9 cells were incubated for 24 h in fresh serum-free DMEM containing diluent or 5 mM azide. P denotes the precursor form of SREBP-2 (~121 kDa) and N denotes the nuclear form of SREBP-2 (~68 kDa). **B**: The abundance of pSREBP-2 (precursor form of SREBP-2) and nSREBP-2 (nuclear form of SREBP-2) in azide-treated cells was compared to that in control cells. The experiment was repeated three independent times and the results were averaged. * Denotes $P < 0.05$ compared to the abundance of each protein in control cells.

We and others have previously shown that AMPK activity is stimulated in azide-treated Clone 9 cells [Abbud et al., 2000; Barnes et al., 2002]. The stimulation of AMPK is related to the rapid rise in cell AMP/ATP ratio following exposure to azide [Jing and Ismail-Beigi, 2006]. This raises the possibility that the observed reduction in the abundance of mRNA encoding cholesterol biosynthetic enzymes is mediated by stimulation of AMPK. We hence determined whether the observed decrease in the abundance of mRNAs encoding the enzymes of cholesterol synthesis by azide can be replicated by exposing Clone 9 cells to AICAR (Table V). Using real-time PCR, we found that the abundance of these mRNAs was not decreased, and in many instances increased. Importantly, the abundance of SREBP-2 transcript that decreased 4.0-fold in response to treatment with azide showed no change in response to treatment with AICAR, and the abundance of SREBP-1c mRNA increased 2.5-fold in AICAR-treated cells. These results clearly suggest that the decrease in expression of mRNAs encoding enzymes of cholesterol synthesis and that of SREBP-2 mRNA following treatment with azide is not mediated by stimulation of AMPK activity.

DISCUSSION

Organisms and cells undergo a myriad of changes as they adapt to new external and internal conditions. In our previous studies on the adaptation of cells in response to inhibition of oxidative phosphorylation by "Chemical hypoxia" or to decreased oxygen tension we have noted that cell ATP levels fall acutely but return to normal levels (within 60 min) despite the continued presence of the inhibitory stimulus for more than 24 h [Mercado et al., 1989; Shetty et al., 1992, 1993b]. Although it is known that during this process some pathways are inhibited while others are stimulated [Ismail-Beigi, 1993; Shetty et al., 1993a], the set of genes involved in this adaptive response remain unknown. In our attempt to study this issue in a systematic and encompassing fashion, we have performed gene microarray analysis of control Clone 9 cells and cells exposed to 5 mM azide for 24 h. Among the few hundred mRNAs whose abundance were either increased or decreased, we made the novel discovery that the abundance of many of the mRNAs encoding enzymes of cholesterol synthesis is decreased. These findings were verified by real-time PCR. We also measured changes in the abundance of SREBP-2 mRNA which was not present in the microarray chip employed. We found that the abundance of SREBP-1c and SREBP-2 mRNAs were also significantly depressed (Tables II and III). It is important to point out that the above changes are not simply due to a fall in cell ATP level, because cell ATP returns to normal within an hour of exposure to azide [Shetty et al., 1992, 1993b; Jing and Ismail-Beigi, 2006], and remains at normal levels for up to 24 h despite the presence of the inhibitor [Shetty et al., 1992]. Moreover, as noted, the abundance of few hundred mRNAs is increased.

The available data indicates that the widely expressed SREBP proteins play a critical role in the transcriptional regulation of enzymes involved in control of cellular lipid synthesis and uptake. SREBP-1a regulates the transcription of genes encoding enzymes of fatty acid and

TABLE V. Effect of AICAR on Relative mRNA Content of Specific Genes Encoding Cholesterol Synthesis by Real-Time RT-PCR

Gene name	Gene symbol	Fold-change ± SE
Cholesterol biosynthesis		
HMG-CoA synthase	Hmgcs1	2.4 ± 0.3
HMG-CoA reductase	Hmgcr	2.3 ± 0.3
Mevalonate kinase	Mvk	2.3 ± 0.2
Lipid metabolism related transcription factors		
Sterol regulatory element binding protein-1c	SREBP-1c	2.5 ± 0.3
Sterol regulatory element binding protein-2	SREBP-2	0.7 ± 0.05
Glucose transporter		
Glut1	Glut1	2.7 ± 0.5

Total RNA was isolated from duplicate or triplicate control Clone 9 cells and cells exposed to 0.1 mM AICAR for 24 h. In each experiment, the relative abundance (fold-change) of the mRNA in AICAR-treated cells was divided by the mean value of control samples. The experiment was repeated 3 to 4 times and the results were averaged (mean ± SE). Controls averaged 1.0 ± 0.1. The number of samples for each mRNA was 6 to 11. All changes, except for SREBP-2, were significant ($P < 0.05$).

cholesterol synthesis, whereas SREBP-1c predominantly, and SREBP-2 near-exclusively, control the transcription of the set of genes encoding enzymes of cholesterol synthesis [Brown and Goldstein, 1997; Horton et al., 2002; Eberle et al., 2004]. The "precursor" forms of SREBPs reside in the ER in non-covalent association with sterol cleavage activating protein (SCAP) a cholesterol binding protein [Horton et al., 2002] and Insigs, ER membrane proteins that serve as anchors of the SREBP-SCAP complex [Gong et al., 2006]. Under conditions of cholesterol excess, cholesterol binds to sterol sensing domain of SCAP, and the Insig-SCAP-SREBP complex is retained in the ER. A deficiency in the cellular content of cholesterol decreases the binding of SCAP to Insig and leads to the translocation of SCAP-SREBP-2 complex to the Golgi apparatus with subsequent cleavage of SREBP-2 by s1p and s2p proteases. This releases the non-membrane bound "nuclear" fragment of SREBP-2 (nSREBP-2) which acts as transcription factor [Brown and Goldstein, 1997; Goldstein et al., 2002]. Interestingly, nSREBP-2 positively controls the transcription of not only the set of genes encoding the enzymes of cholesterol synthesis, Insig-1 (11) but also the transcription of SREBP-2 gene itself [Sato et al., 1996]. Our results show that the abundance of SREBP-2 and Insig-1 mRNAs as well as the abundance of both pSREBP-2 and nSREBP-2 are decreased in cells exposed to azide, raising the possibility that the decrease in SREBP-2 expression and/or function might well mediate the observed decrease in the abundance of the set of mRNAs encoding enzymes of cholesterol synthesis, and the consequent inhibition of cholesterol synthesis observed under these conditions. It should be noted that the abundance of nSREBP-2 was decreased to a lesser extent than that of pSREBP-2 suggesting that the activity of nSREBP-2 may have also decreased. For example, nSREBP-2 function may be suppressed via sumoylation [Hirano et al., 2003] or through binding of an antagonistic transcription factor, such as ATF-6 [Zeng et al., 2004]. It is also possible that conversion of pSREBP-2 to nSREBP-2 is the major pathway of pSREBP-2 turnover. In such a scenario, the precursor pool might become depleted prior to depletion of the nSREBP-2 pool. Further detailed studies are needed to test these possibilities.

The difference in the magnitude of decrements in mRNA abundance of the various cholesterol synthetic enzymes, including that of SREBP-2 mRNA, deserves comment. The abundance of each mRNA is controlled by its specific rate of synthesis and degradation. If synthesis is markedly reduced, then the abundance of each mRNA will decrease based on its rate of degradation. Which itself may be varied with the treatment. Hence, it is not possible to predict the changes in the abundance of the mRNAs that can be directly attributed to a decrease in nSREBP-2 content.

We also examined the possibility that the decreased abundance of mRNAs encoding enzymes of cholesterol synthesis in azide-treated cells is mediated by stimulation of AMPK. In these experiments, AICAR was employed as a stimulator of AMPK [Corton et al., 1995]. Remarkably, and in contrast to the effect of azide, the abundance of three mRNAs encoding enzymes of cholesterol synthesis were modestly increased in AICAR-treated cells (Table V); the abundance of SREBP-1c and Glut1 mRNAs were also increased. While these novel findings require further study in their own right, they strongly suggest that the effect of azide on the expression of these mRNAs is not mediated by stimulation of AMPK. A recent report indicated that hypoxia causes an upregulation of SREBP-2 in S. Pombe [Hughes et al., 2005], while we noted a decrease in SREBP-2 mRNA in response to azide and no change in response to AICAR. The divergent results may be due to the different experimental systems employed. It should also be noted that hypoxia results in inhibition of a number of cellular processes in addition to inhibition of oxidative phosphorylation.

We also noted that the rate of cholesterol synthesis in azide-treated cells was $\sim$1/10th of control cells, a value that is significantly lower than some of the decreases in mRNAs including that encoding HMG-CoA reductase. It is well known that stimulation of AMPK will lead to phosphorylation and inactivation of HMG-CoA reductase [Henin et al., 1995]. Our previous finding that AMPK is stimulated in azide-treated cells [Abbud et al., 2000; Jing and Ismail-Beigi, 2006] suggests that inactivation of HMG-CoA reductase is probably also contributing to inhibition of cholesterol synthesis.

The finding of a decrease in the abundance of mRNAs encoding enzymes of cholesterol

synthesis and that of SREBP-2 mRNA and protein in azide-treated cells in the face of decreased cell cholesterol content is a novel and striking finding. Specifically, conditions that lead to a decrease in the cellular cholesterol content such as following inhibition of HMG-CoA reductase by statins or interference with cellular uptake of cholesterol lead to an increase in the content of the nSREBP-2 in the nucleus resulting in enhanced transcription of the set of genes encoding the cholesterol synthetic pathway [Sato et al., 1996; Brown and Goldstein, 1997; Shimomura et al., 1997], and thereby increasing the abundance of these mRNAs and an increase in cholesterol synthesis. In sharp contrast, our results show that the decrease in cell cholesterol content, decreased cholesterol synthesis in azide-treated cells is associated with *decreased* abundance of the above mRNAs, and decreased p- and nSREBP-2 contents, presumably through transcription and/or translational mechanisms. These findings strongly suggest that the stimulus and the regulatory mechanisms that control the expression of this set of genes in azide-treated cells are strong enough to overcome completely the well-described upregulation of these genes by decreases in cellular content of cholesterol. This premise is in keeping with the recent observation that a decrease in Insig expression and/or in cell cholesterol increases the stability of HMG-CoA reductase and leads to an increased rate of cholesterol synthesis [Engelking et al., 2005]. Elevated cell cholesterol level triggers the binding of HMG-CoA reductase to Insigs and thereby initiates the process of ubiquitination and degradation of HMG-CoA reductase. Under conditions of low sterols, HMG-CoA reductase exhibits an extended lifetime leading to an increase in the abundance of the active enzyme leading to increased cholesterol synthesis. The decrease in the abundance of SREBP-2 mRNA and nSREBP-2, however, may help explain the decrease in the abundance of the set of mRNAs encoding enzymes of cholesterol synthesis, and the subsequent inhibition of cholesterol synthesis. The decrease in the synthesis of cholesterol cannot be simply attributed to profound changes in cell ATP, since cell ATP, ADP, and AMP levels return to normal levels after 1 h of incubation in the presence of azide [Jing and Ismail-Beigi, 2006], and cell ATP levels remain at normal levels for 24 h [Shetty et al., 1992]. Further studies are required to explore the mechanisms underlying the control of cholesterol synthesis following inhibition of oxidative phosphorylation by azide, and the role of decreased expression of HMG-CoA reductase and SREBP-2 transcripts and SREBP-2 protein levels in the above response require further investigation.

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