Mitochondrial Reactive Oxygen Species Signal Hepatocyte Steatosis by Regulating the Phosphatidylinositol 3-Kinase Cell Survival Pathway

Rohit Kohli, Xiaomin Pan, Padmini Malladi, Mark S. Wainwright, and Peter F. Whittington

From the Department of Pediatrics, Children's Memorial Hospital, Children's Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60614

Abnormal dietary intake of macronutrients is implicated in the development of obesity and fatty liver disease. Steatosis develops in cultured hepatocytes exposed to medium containing either a high concentration of long chain free fatty acids (HFFA) or medium deficient in methionine and choline (MCD). This study examined the mitochondrial reactive oxygen species (ROS)-dependent regulation of the phosphoinositol (PI) 3-kinase pathway in steatosis induced by exposure of AML-12 mouse hepatocytes to MCD or HFFA medium. Exposure to either MCD or HFFA medium resulted in increased production of superoxide anions and \( \text{H}_2\text{O}_2 \), transduction of the PI 3-kinase pathway and steatosis. Inhibition of PI 3-kinase with LY294002 prevented steatosis. Pharmacologically inhibiting electron transport chain complex III production of ROS prevented activation of PI 3-kinase during macronutrient perturbation, whereas pharmacologically promoting electron transport chain complex III ROS production activated PI 3-kinase independent of nutrient input. The data suggest that \( \text{H}_2\text{O}_2 \) is the ROS species involved in signal transduction; promoting the rapid conversion of superoxide to \( \text{H}_2\text{O}_2 \) does not inhibit PI 3-kinase pathway activation during nutrient perturbation, and exogenous \( \text{H}_2\text{O}_2 \) activates it independent of nutrient input. In addition to transducing PI 3-kinase, the ROS-dependent signal cascade amplifies the PI 3-kinase signal by maintaining phosphatase and tensin homolog in its inactive phosphorylated state. Knockdown of phosphatase and tensin homolog by small interfering RNA independently activated the PI 3-kinase pathway. Our findings suggest a common path for response to altered nutrition involving mitochondrial ROS-dependent PI 3-kinase pathway regulation, leading to steatosis.

Innumerable epidemiologic studies demonstrate that inappropriate intake of macronutrients and a sedentary lifestyle conspire to produce rampant obesity in the United States. Hepatic steatosis (the accumulation of excess neutral fat within hepatocytes) in obese persons is thought to result from metabolic disturbances attendant to obesity. An estimated 30 million adults and 1.6 million children in the United States have nonalcoholic fatty liver disease (NAFLD), of which an estimated 30% have nonalcoholic steatosis (NASH), which can lead to chronic debilitating liver injury, including cirrhosis (1). Hepatic steatosis is the defining feature of NAFLD (2). It is commonly held that steatosis is the precursor of events that lead to the transition of NAFLD to NASH, including the induction of oxidative stress and activation of inflammatory pathways (3, 4). The development of hepatic steatosis is therefore considered to be the necessary first effect of disturbed nutrition that induces the cascade of events leading to NASH. How disturbed nutrition causes hepatic steatosis is not well understood.

Experimental NASH can be produced in laboratory rodents by dietary manipulation, including both restricted intake (i.e. methionine-choline deficient (MCD) diet) (5) and excess intake (i.e. various high fat diets) (6, 7). How nutritional excess and deprivation can both culminate in experimental NASH is not clear (8).

The MCD diet-fed mouse is a well accepted and reliable model of experimental NASH (9, 10). Our findings using this model have suggested that this is not so much a model of toxicity induced by deficiency of methyl donor molecules, as is commonly thought, but a model of dietary imbalance that incites an orchestrated response leading to experimental NASH. In a detailed time course study of female A/J mice fed the MCD diet, we found that these mice developed significant steatosis and massive overexpression of the Th1 cytokine osteopontin (OPN) within 1 week of starting the diet (11). The hepatic expression of OPN protein was 16-fold increased after 1 day of diet treatment and 40-fold increased after 2 days, whereas mRNA expression was not increased until 2 weeks, suggesting translational regulation of early OPN protein expression. These events occurred at a time when the diet is unlikely to have produced toxic deficiency. Furthermore, no oxidative injury developed until 4–8 weeks of dietary depriva-

---

1 To whom correspondence should be addressed: Children’s Memorial Research Center, 2300 Children’s Plaza, Chicago, IL 60614. Tel.: 773-880-4643; Fax: 773-975-8671; E-mail: p-whittington@northwestern.edu.

2 The abbreviations used are: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatosis; MCD, medium deficient in methionine and choline; HFFA, medium containing a high concentration of long chain free fatty acids; PI, phosphatidylinositol; OPN, osteopontin; ROS, reactive oxygen species; PTEN, phosphatase and tensin homolog; pPTEN, phosphorylated PTEN; pIP3, inositol 1,4,5-triphosphate; PBS, phosphate-buffered saline; DHE, dihydroethidium; DCF, 2',7'-dichlorofluorescin diacetate; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin; siRNA, small interfering RNA; ETC, electron transport chain; FFA, free fatty acids(s).
Mitochondrial ROS Signal Steatosis via PI 3-Kinase

mitigation. Therefore, it seems reasonable to conclude that the dietary change directly influenced the induction of the metabolic response represented by OPN overexpression.

To further explore the possibility that the MCD diet represented a signal leading to steatosis and other expressions of experimental NASH, including the overexpression of OPN, we examined the effects of substituting medium deficient in methionine and choline (MCD medium) on AML-12 mouse hepatocytes in vitro (12). Substituting MCD medium for control medium produced significant hepatocyte steatosis by 18 h of exposure. Exposure to MCD medium induced significant increases in OPN protein expression by 6 h of treatment. Thus, the in vitro situation mimicked the early events in the in vivo MCD diet model. We further showed that exposure to the MCD medium induced a significant increase in the PI 3-kinase p85 regulatory subunit expression beginning at 1 h of incubation and a sustained increase in pAkt expression during 1–18 h of incubation. Inhibiting PI 3-kinase with LY294002 blocked the MCD medium-induced increases of OPN expression and prevented cellular steatosis from developing. Taken together, these findings suggested that there is an orchestrated cellular response to the stress induced by exposure to MCD medium in which OPN expression and steatosis are dependent upon the activation of the PI 3-kinase pathway.

In the present study, we sought to determine the mechanisms by which nutrient perturbation produces steatosis in hepatocytes in culture. Feldstein et al. (13) demonstrated that human hepatocytes (HepG2 cells) in culture treated with medium containing excess long-chain fatty acid (HFFA) medium develop steatosis. We hypothesized that nutrient perturbation in the form of macronutrient deprivation (MCD medium) or excess (HFFA medium) activates and modulates the PI 3-kinase cell signaling pathway to produce steatosis. We proposed that its activation results from increased production of mitochondrial reactive oxygen species (ROS) induced by the change in macronutrient input. Mitochondrial dysfunction and injury are thought to be important in the pathogenesis of NAFLD (14–18). Mitochondria are a major source of ROS in cells (19). ROS are produced by mitochondria during normal oxidative metabolism, and we hypothesized that a change in macronutrient input could induce a metabolic stress that would increase the rate of ROS production by mitochondria. ROS species, in particular H₂O₂, can act as signals by increasing tyrosine phosphorylation of receptor tyrosine kinases even in the absence of growth factor binding, thus independently inducing the activation of downstream signaling cascades like that of PI 3-kinase (20). Showing that perturbation of macronutrient input to hepatocytes, either deprivation or excess input, produces steatosis by mechanisms that are dependent upon mitochondrial ROS regulating the PI 3-kinase pathway would provide evidence for a novel link between abnormal dietary intake and NASH.

We further proposed that phosphatase and tensin homolog (PTEN) is mechanistically involved in the positive modulation of the PI 3-kinase signaling pathway during nutrient perturbation. PTEN is the natural inhibitor of the PI 3-kinase pathway, acting to degrade PIP₃ produced by PI 3-kinase before it can activate Akt through phosphorylation. PTEN can be inactivated by exposure to ROS. Specifically, exposure of purified PTEN or cells to H₂O₂ can result in time and concentration-dependent oxidation of PTEN and reduction of phosphatase activity (21). Further, PTEN function is regulated by its membrane interaction, which is determined in large part by the degree of phosphorylation of the C-terminal tail (22). The constitutively phosphorylated protein (pPTEN) must be dephosphorylated before it can bind to membrane PDZ-containing proteins and assume full functionality as a lipid phosphatase (23, 24). However, the dephosphorylated protein is unstable and rapidly degraded (23). Thus, the phosphatase activity of PTEN is entirely dependent upon the rate of dephosphorylation of pPTEN, providing tight regulation of this important suppressor of the PI 3-kinase/Akt pathway. PTEN activity is modulated in this way during ischemia/perfusion stress (25), which suggests that ROS signaling may be involved. Finally, the importance of PTEN in hepatic steatosis was demonstrated by showing that liver-specific PTEN knockouts develop steatosis and NASH-like disease (26). If nutrient perturbation activates PI 3-kinase and inhibits PTEN function, it would provide evidence of a novel signal amplification mechanism with potential importance in the evolution of fatty liver disease.

The overall aim of the present study was to determine the signaling mechanisms involved in the development of hepatocyte steatosis resulting from altered macronutrient input, specifically the role of mitochondrial ROS in the downstream activation and modulation of the PI 3-kinase pathway.

EXPERIMENTAL PROCEDURES

Hepatocyte Cell Culture—AML-12 cells, a mouse hepatocyte cell line that has been stably transfected with human transforming growth factor-α (American Type Culture Collection, Manassas, VA), were treated as previously described (12). In brief, these cells are grown to 70–80% confluence in Dulbecco’s modified Eagle’s medium/Ham’s nutrient mixture F-12 containing 10% fetal bovine serum (for growth factors) and then made quiescent by incubating for 24 h in standard medium without fetal bovine serum. Quiescent cells were then exposed either to control growth medium (MC; methionine (0.116 mM) and choline (0.064 mM)) or identical medium that was manufactured to be completely deficient in methionine and choline (MCD medium) purchased from Invitrogen. In separate experiments, cells were treated with control medium and HFFA medium (addition of 1.0 mmol/liter fatty acid mixture of 2:1 oleate/palmitate) with 1% bovine serum albumin supplemented with identical glucose and serum concentrations. This HFFA construct was used because it has been successfully used in vitro to produce steatosis and experimental NASH in cultured primary mouse hepatocytes and HepG2 cells (13). Cells were then maintained at 37 °C for various times before harvesting for analysis. Inclusion of pharmacologic agents with control or test media was used as described under “Results.”

Steatosis Analysis—Cellular triglyceride content was determined after 18 h of treatment as described (12). Cells were scraped from culture plates that had been rinsed with cold PBS and were subsequently pelleted by centrifugation. The cell pellet was suspended in 20 mM Tris buffer, pH 7.5, and homogenized. Cell triglyceride content in the whole homogenates was

21328 JOURNAL OF BIOLOGICAL CHEMISTRY
measured using a spectrophotometric kit procedure (Thermo 
DMA, Arlington, TX) and expressed as μg of triglyceride/mg of 
protein. Hepatocyte steatosis was also determined in situ using 
standard Oil-Red O dye staining.

Confocal Time Lapse Microscopy for Analysis of ROS— 
AML-12 cells grown on glass bottom Petri dishes (Matek, Ash-
land, MA) and made quiescent were exposed to control 
medium containing 4',6-diamidino-2-phenylindole (DAPI) 
dye (Sigma) for 20 min, rinsed with sterile PBS once, and then 
incubated with control medium containing the ROS-sensitive 
dyes dihydroethidium (DHE) (Invitrogen) or 2',7'-dichlo-
rofluorescin diacetate (DCF) (Sigma) in PBS for 30 min at 37 °C. 
Dishes were once again rinsed with PBS and subsequently 
exposed to control and test media containing 10% serum and 
DHE. Some experiments were done in the presence of the 
superoxide dismutase Mn(III) tetrakis (4-benzoic acid) porphy-
rin (MnTAP) (A. G. Scientific, San Diego, CA) or electron 
transport chain (ETC) complex III inhibitors stigmatellin, myx-
othiazol, or antimycin A (Sigma). In some experiments, 
AML-12 cells were exposed to exogenous H2O2 (25 μM or 1.0 
mm) by its addition to the medium. For experiments involving 
oxidation of PTEN, we chose 1 mM H2O2 as the lowest con-
centration resulting in nearly maximal oxidation of PTEN when 
applied to HeLa and NIH 3T3 cells (21). Preliminary studies 
showed this concentration to not be lethal to AML-12 cells. If 
experiments were extended beyond 15 min, H2O2 was replen-
ished by injection every 15 min to maintain the concentration 
>1 mM. Real time fluorescent images were obtained using a 
Zeiss 510 META confocal laser-scanning microscope while 
maintaining dishes at 37 °C.

pPTEN Immunofluorescence—AML-12 cells were grown and 
treated as described above for confocal microscopy with the 
exception that DAPI and DHE were not included. Cells were 
treated for 30 min with control and test media and then fixed in 
situ using 4% paraformaldehyde and washed with 0.2% Triton 
X-100 followed by PBS. This was followed by incubation at 
room temperature for 1 h with 3% bovine serum albumin and 
overnight incubation with the primary pPTEN (Ser380/Thr382/
/Thr386) antibody (Cell Signaling, Danvers, MA) at 4 °C, fol-
lowed by incubation with an anti-rabbit IgG fluorescein iso-
thiocyanate antibody and DAPI. Fluorescence was recorded 
with green (fluorescein isothiocyanate antibody) and blue (DAPI) filters 
using an upright LEICA DMR-HC microscope.

Western Blot Analysis—Immunoreactive levels of various 
proteins and phosphoproteins were assessed by Western blot 
analysis. Cell homogenates containing 10–25 μg of protein 
were resolved by 7.5–10% SDS-polyacrylamide gel electro-
phoresis and transferred overnight onto nitrocellulose mem-
branes by electrophoresis. Specific primary antibodies to PI 
3-kinase p85 subunit, pAkt1 (Ser473), and Akt2 (Upstate Bio-
technology, Lake Placid, NY); Akt, pAkt, PTEN, and pPTEN 
(Cell Signaling, Danvers, MA); and Akt1 (Chemicon, Temecula, 
CA) were used to assess their expression. β-Actin antibodies 
were used to confirm equal protein loading among samples; 
these results always showed equal loading and are not shown.
The bound primary antibodies were detected with a respective 
peroxidase-conjugated secondary antibody (1:1000 dilution; 
Amersham Biosciences) and visualized with an enhanced 
chemiluminescence method. Protein expression 
levels were determined by densitometric analysis.

PTEN siRNA—AML-12 cells at 60–75% confluence were 
transfected with a range of concentrations of small interfering 
RNA (siRNA) targeted against PTEN (target sequence ATC 
GAT AGC ATT TGC AGT ATA) using HiPerFect transfection 
reagent and protocol (Qiagen, Cambridge, MA). After 18 h, 
cells were harvested, and total cellular RNA was purified. The 
RNA was then reverse transcribed, and the resultant cDNA 
was used for quantitative real time RT-PCR for PTEN (primers 
available upon request). In separate experiments, total cellular 
protein was purified and used to load Western blots as 
described above.

Statistical Analysis—Comparison between groups was 
performed using Student’s t test for unpaired samples or by 
analysis of variance. A p value of <0.05 was considered statistically 
significant.

RESULTS

Treating with MCD or HFFA Medium Produces Hepatocyte 
Steatosis—Treating cultured AML-12 hepatocytes with MCD 
or HFFA medium for 18 h resulted in steatosis, as demonstrated 
by Oil-Red O staining (Fig. 1A). Average cell triglyceride con-
tent was significantly increased from control by treatment for 
18 h with MCD (p < 0.05 versus control) and HFFA media (p < 
0.01 versus control) (Fig. 1B). Cell viability as determined by 
trypan blue exclusion was not reduced by incubating with 
either MCD or HFFA medium for 18 h (data not shown).

PI 3-Kinase/Akt Pathway Transduction Is Necessary for 
Development of Hepatocyte Steatosis during Treatment with 
MCD or HFFA Medium—We previously demonstrated the 
avtication of the PI 3-kinase pathway in AML-12 cells exposed 
to MCD medium with maximal activation at 1 h of exposure 
and that steatosis was prevented by treating cells exposed to 
MCD medium with LY294002 (12). In the present study, 
Western blot analysis of cell homogenates with specific antibodies
against the p85 regulatory subunit and pAkt demonstrated transduction of the PI 3-kinase pathway to a similar degree after 1 h of exposure to either MCD or HFFA medium (Fig. 2A). The isoforms of Akt, Akt1 and Akt2, have distinct roles in regard to growth and glucose homeostasis (27). Their relative importance in liver metabolism is not well known. To determine which Akt isoform is involved in this signaling cascade, we used specific Akt1 and Akt2 antibodies to demonstrate that Akt1 is the predominant Akt isoform in AML-12 cells and show that its expression does not change with exposure to nutrient stress. Increased expression of pAkt1 after exposure to macronutrient perturbation parallels the increased expression of pAkt. C, preventing PI 3-kinase/Akt signal transduction with the specific PI 3-kinase inhibitor LY294002 (LY) abrogates the steatosis produced by macronutrient perturbation. Treatment with 10 μM LY (LY10) completely eliminates the increase in cellular triglyceride content produced by MCD treatment, whereas LY294002 at 10–50 μM concentration markedly reduces the increase produced by HFFA. These experiments provide confirmatory data on the involvement of the PI 3-kinase cell survival pathway in the development of steatosis resulting from macronutrient perturbation. ∗, p < 0.05.

**FIGURE 2.** Transduction of the PI 3-kinase/Akt pathway during exposure to MCD and HFFA medium and dependence of steatosis upon PI 3-kinase. A, Western blots for p85 and pAkt in AML-12 cells exposed to either HFFA or MCD medium for 1 h show increased p85 expression and increased phosphorylation of Akt relative to control cells. These blots show that either macronutrient perturbation resulted in activation of the PI 3-kinase cell survival pathway. B, Western blots for Akt1 and Akt2 in AML-12 cells exposed to control, HFFA, or MCD medium for 1 h show Akt1 to be the predominant Akt isoform in AML-12 cells and show that its expression does not change with exposure to nutrient stress. Increased expression of pAkt1 after exposure to macronutrient perturbation parallels the increased expression of pAkt. C, preventing PI 3-kinase/Akt signal transduction with the specific PI 3-kinase inhibitor LY294002 (LY) abrogates the steatosis produced by macronutrient perturbation. Treatment with 10 μM LY (LY10) completely eliminates the increase in cellular triglyceride content produced by MCD treatment, whereas LY294002 at 10–50 μM concentration markedly reduces the increase produced by HFFA. These experiments provide confirmatory data on the involvement of the PI 3-kinase cell survival pathway in the development of steatosis resulting from macronutrient perturbation. ∗, p < 0.05.

**FIGURE 3.** Production of superoxide anions by AML-12 hepatocytes exposed in culture to either MCD or HFFA medium. Time lapse images were captured by confocal fluorescence microscopy staining for nuclei (blue, DAPI stain) and superoxide (red, DHE stain). A, the superoxide content of cells exposed to control medium does not change over 30 min of incubation. B, the superoxide content of liver cells increases significantly when exposed to MCD medium for 30 min. C, the superoxide content of liver cells increases significantly when exposed to HFFA medium for 30 min. The effect seems to be somewhat greater than that seen with MCD medium, although direct quantitative comparisons are difficult. D, the increase in superoxide content seen after exposure to HFFA medium is decreased significantly when similar cells are co-treated with the exogenous superoxide dismutase MnTBAP (50 μM). Similar findings in cells exposed to MCD medium and MnTBAP are not shown.

Treating with MCD or HFFA Medium Increases Hepatocyte Superoxide Production—We hypothesized that altered macronutrient input could produce metabolic stress that would stimulate hepatocyte mitochondria to increase ROS production. The ROS-sensitive vital dye DHE was used to detect the production of superoxide in real time using live cell confocal time lapse fluorescence microscopy. Incubating with either MCD or HFFA medium produced marked increases in red DHE fluorescence within 30 min (Fig. 3, A–C) relative to control medium, indicating that either change in nutritional milieu prompted an increase in ROS generation.

**H₂O₂ Is Probably Involved as a Signal Molecule in Transduction of the PI 3-Kinase/Akt Pathway—**Superoxide is normally consumed in the production of H₂O₂ by the action of superoxide dismutase, and in contrast to superoxide, H₂O₂ is readily permeable across the mitochondrial membrane. To determine if H₂O₂ could be the signal emanating from mitochondria stimulated by nutrient perturbation, cells treated with either MCD or HFFA medium were co-incubated with the exogenous MnTBAP (50 μM). Similar findings in cells exposed to MCD medium and MnTBAP are not shown.

**FIGURE 3.** Production of superoxide anions by AML-12 hepatocytes exposed in culture to either MCD or HFFA medium. Time lapse images were captured by confocal fluorescence microscopy staining for nuclei (blue, DAPI stain) and superoxide (red, DHE stain). A, the superoxide content of cells exposed to control medium does not change over 30 min of incubation. B, the superoxide content of liver cells increases significantly when exposed to MCD medium for 30 min. C, the superoxide content of liver cells increases significantly when exposed to HFFA medium for 30 min. The effect seems to be somewhat greater than that seen with MCD medium, although direct quantitative comparisons are difficult. D, the increase in superoxide content seen after exposure to HFFA medium is decreased significantly when similar cells are co-treated with the exogenous superoxide dismutase MnTBAP (50 μM). Similar findings in cells exposed to MCD medium and MnTBAP are not shown.

Treating with MCD or HFFA Medium Increases Hepatocyte Superoxide Production—We hypothesized that altered macronutrient input could produce metabolic stress that would stimulate hepatocyte mitochondria to increase ROS production. The ROS-sensitive vital dye DHE was used to detect the production of superoxide in real time using live cell confocal time lapse fluorescence microscopy. Incubating with either MCD or HFFA medium produced marked increases in red DHE fluorescence within 30 min (Fig. 3, A–C) relative to control medium, indicating that either change in nutritional milieu prompted an increase in ROS generation.
Mitochondrial ROS Signal Steatosis via PI 3-Kinase

Antimycin A is a complex III inhibitor that paradoxically increases superoxide production (28). Treating AML-12 cells in control medium with antimycin A for 30 min increased DHE fluorescence (Fig. 6A) and produced a dose-dependent increase in p85 and pAkt expression to a similar degree as nutrient perturbation (Fig. 6C). To determine whether the increased ROS production induced by treatment with antimycin A could produce steatosis in the absence of nutrient perturbation, AML-12 cells grown in control medium were exposed to very low dose (10–20 nM) antimycin A (necessary because of its toxicity at higher dose) for 5 days, which resulted in steatosis (Fig. 6B). Taken together, these data indicate the source of increased superoxide production resulting from changes in nutrient input to be largely complex III of the ETC.

PTEN Is Inactivated during Nutrient Perturbation, Leading to Positive Modulation of the PI 3-Kinase/Akt Pathway—PTEN is a lipid phosphatase naturally involved in inhibiting the PI 3-kinase pathway by degrading PIP3. PTEN is constitutively phosphorylated, and failure to dephosphorylate it leaves it inactive and unable to effectively degrade PIP3 (24). Exposure of PTEN to H2O2 can oxidize it, which also reduces its ability to degrade PIP3 (21). If exposure to MCD or HFFA resulted in either of these changes in PTEN, it would result in loss of inhibition of the PI 3-kinase/Akt pathway by preventing PIP3 degradation. AML-12 cells exposed to MCD or HFFA medium expressed substantial increases in cellular pPTEN, as demonstrated by immunofluorescence microscopy (Fig. 7A) and Western blot (Fig. 7B). We were able to demonstrate oxidation of PTEN when cells were exposed to 1 mM H2O2, but not with either nutrient treatment (Fig. 7C). These findings suggest that in this setting, phosphorylation is the main mechanism of control medium with inclusion of H2O2 demonstrated an increase in p85 and pAkt expression to a similar degree as with nutrient perturbation (Fig. 4D). These data show that accelerating the conversion of superoxide to H2O2 does not reduce the effect of nutrient change on PI 3-kinase activation, H2O2 is rapidly generated and distributed to the cytoplasmic compartment with either nutrient change, and H2O2 can initiate transduction of the PI 3-kinase pathway independent of nutrient perturbation.

Mitochondria Are the Source of Increased Superoxide Production Seen with MCD or HFFA Medium—Complexes I and III of the mitochondrial ETC leak superoxide anions during normal respiration. Superoxide is specifically produced in large part by complex III of the mitochondrial ETC. To confirm complex III as the source of superoxide production during acute nutrient perturbation, cells exposed to either MDC or HFFA medium were treated with specific ETC complex III inhibitors stigmatellin and myxothiazol, both of which decrease mitochondrial superoxide production. As expected, the increase in DHE fluorescence with HFFA exposure was abrogated by treatment with either compound (Fig. 5, A–C). Similarly, treatment with either compound eliminated the increase in DHE fluorescence with exposure to MCD medium (data not shown). Moreover, both ETC complex III inhibitors eliminated the increased expressions of p85 and pAkt that were produced by either nutrient perturbation alone (Fig. 5, D–F).

3D), indicating the rapid elimination of superoxide in the formation of H2O2. However, transduction of the PI 3-kinase pathway was not altered in cells exposed to either MCD or HFFA medium along with the permeable superoxide dismutase mimic MnTBAP as evidenced by no reduction in expression of p85 and pAkt (Fig. 4, A and B). Thus, signal transduction with either nutrient perturbation was unaffected by the rapid conversion of superoxide to H2O2. The ROS-sensitive vital dye DCF was used to detect the production of H2O2 in real time using live cell confocal time lapse fluorescence microscopy. Incubating with either MCD or HFFA medium produced marked increases in green DCF fluorescence within 10 min of exposure (Fig. 4C) relative to control medium and similar to cells exposed to exogenous H2O2, indicating that either change in nutritional milieu prompted an increase in H2O2 production. The H2O2 appeared to be distributed throughout the cytoplasm of the cells, as evidenced by the distribution of fluorescence (Fig. 4C), and persisted for at least 30 min after exposure, when experiments were terminated (data not shown). Cells grown in

FIGURE 4. Evidence for a role of H2O2 in signaling PI 3-kinase transduction in AML-12 hepatocytes exposed in culture to nutritional stress. A, Western blot shows increased p85 and pAkt expression in cells exposed to MCD medium for 1 h relative to control. Treatment with the superoxide dismutase MnTBAP (25 μM) did not reduce p85 and pAkt expression resulting from exposure to MCD medium. B, treatment with HFFA medium for 1 h results in increased p85 and pAkt expression in cells relative to control. Treatment with MnTBAP (25 μM) did not reduce p85 and pAkt expression resulting from exposure to HFFA medium. C, images were captured by confocal fluorescence microscopy staining for nuclei (blue, DAPI stain) and H2O2 (green, DCF stain). Cells exposed to either MCD or HFFA medium showed increased H2O2 generation far in excess of cells treated with control medium and DCF staining equivalent to that of cells treated with exogenous 25 μM H2O2. D, Western blots for p85 and pAkt in cells exposed to exogenous H2O2 (25 μM) for 1 h show increased p85 expression (~1.5 times control) and massively increased pAkt expression relative to untreated cells.
PTEN inactivation. This could be the result of increased active phosphorylation or failure to dephosphorylate. They also suggest that the functional intracellular concentration of H$_2$O$_2$ produced by nutrient treatment is less than that resulting from exposure to 1 mM exogenous H$_2$O$_2$. To demonstrate that PTEN functions to inhibit the PI 3-kinase pathway specifically in hepatocytes, AML-12 cells cultured in control medium were transfected with siRNA against PTEN. This treatment resulted in dose-dependent reduction in PTEN mRNA expression and activation of the PI 3-kinase pathway, as evidenced by increased cellular concentration of pAkt (Fig. 8, A–C). Taken together, these data indicate that PTEN functions in hepatocytes to inhibit the PI 3-kinase pathway and that during nutrient perturbation, the inhibition of PTEN function through maintaining a relatively phosphorylated state potentially provides amplification of PI 3-kinase signaling.

A Critical Threshold Concentration of Free Fatty Acid Is Needed to Produce PI 3-Kinase Signal Transduction—In the foregoing experiments, HFFA was defined as a 1.0 mmol/liter fatty acid mixture of 2:1 oleate/palmitate with 1% bovine serum albumin supplemented with identical glucose and serum concentrations as taken from Feldstein's work (13). We sought to determine exactly what concentration of fatty acid is needed to exert the effect of exposure to HFFA medium and to determine if the effect of increasing FFA concentrations is graded versus a critical threshold concentration to produce the effect. The data produced by nutrient treatment is less than that resulting from exposure to 1 mM exogenous H$_2$O$_2$. To demonstrate that PTEN functions to inhibit the PI 3-kinase pathway specifically in hepatocytes, AML-12 cells cultured in control medium were transfected with siRNA against PTEN. This treatment resulted in dose-dependent reduction in PTEN mRNA expression and activation of the PI 3-kinase pathway, as evidenced by increased cellular concentration of pAkt (Fig. 8, A–C). Taken together, these data indicate that PTEN functions in hepatocytes to inhibit the PI 3-kinase pathway and that during nutrient perturbation, the inhibition of PTEN function through maintaining a relatively phosphorylated state potentially provides amplification of PI 3-kinase signaling.

FIGURE 5. Effect of specific pharmacologic mitochondrial ETC complex III inhibitors on AML-12 hepatocytes exposed in culture to nutritional stress. Myxothiazol and stigmatellin are compounds well known to decrease superoxide production from complex III of the ETC. A, the superoxide content of liver cells as evidenced by red DHE fluorescence increases significantly when exposed to HFFA medium for 30 min. B, co-treatment of AML-12 cells with HFFA medium and the ETC inhibitor myxothiazol (5 $\mu$m) substantially reduces DHE fluorescence compared with cells treated with HFFA alone. C, co-treatment of AML-12 cells with HFFA medium and the ETC inhibitor stigmatellin (5 $\mu$m) substantially reduces DHE fluorescence compared with cells treated with HFFA alone. D, co-treating cells exposed to MCD medium for 1 h with stigmatellin (dose effect 1, 2, and 5 $\mu$m) reduces the increased p85 expression and eliminates the increased pAkt expression seen in cells treated with MCD medium alone. E, a similar effect is seen when co-treating cells exposed to MCD medium for 1 h with myxothiazol (5 $\mu$m). F, similarly, co-treating cells exposed to HFFA medium for 1 h with the ETC inhibitors stigmatellin (5 $\mu$m) or myxothiazol (5 $\mu$m) eliminates the increased p85 and pAkt expressions observed with HFFA medium alone.

FIGURE 6. Treating AML-12 cells with the mitochondrial ETC complex III inhibitor antimycin A mimics the effects of treating with MCD or HFFA medium. Antimycin A is a compound known to inhibit electron transport within complex III of the ETC while paradoxically increasing production of superoxide anions. A, time lapse images captured by fluorescence confocal microscopy staining for nuclei (blue, DAPI stain) and superoxide (red, DHE stain) show that treatment of AML-12 cells with antimycin A over 30 min increases superoxide production. B, neutral fat accumulation after 5 days of exposure to antimycin A is shown by staining of cells with Oil-Red O; both 10 and 20 nM treatment resulted in greater steatosis than control conditions. The difference in magnitude of antimycin A doses between the longer and shorter term experiments was necessitated by need for cell viability over a 5-day period. C, cells treated with antimycin A (0.5–2 $\mu$m) in control medium for 1 h show increased p85 and pAkt expressions in a dose-dependent manner.
shown in Fig. 9 demonstrates that a critical threshold concentration ~0.25 mmol/liter fatty acid mixture of 2:1 oleate/palmitate produces the PI 3-kinase transduction ascribed to HFFA medium.

DISCUSSION

The present study examined the signaling mechanisms involved in the development of hepatocyte steatosis resulting from altered macronutrient input to hepatocytes in culture and resulted in several important findings. 1) Both nutrient deprivation in the form of MCD medium and nutrient excess in the form of HFFA medium resulted in a rapid increase in superoxide production. 2) Either nutrient perturbation resulted in transduction of the PI 3-kinase signaling pathway and steatosis that can be prevented by inhibiting PI 3-kinase. 3) We show through a variety of manipulations that the mitochondrial ETC III is the source of ROS and that H$_2$O$_2$ is the probable ROS signaling the transduction of the PI 3-kinase pathway during macronutrient perturbation. 4) Increasing superoxide production during normal cellular respiration by means other than macronutrient perturbation has the identical effects of transducing the PI 3-kinase pathway and producing steatosis. 5) Inhibition of PTEN through phosphorylation appears to be involved in positive modulation of the PI 3-kinase signal during macronutrient perturbation. These findings link abnormal macronutrient input to a signaled response, leading to hepatocyte steatosis.

We construe the abrupt increase in ROS production during nutrient perturbation to be the result of an acute metabolic stress imposed by the change in macronutrient input. This find-
Mitochondrial ROS Signal Steatosis via PI 3-Kinase

Our data suggest that the signal emanating from the mitochondria involved in signaling the PI 3-kinase/Akt pathway is H$_2$O$_2$. Other data support the idea that ROS-mediated signaling is involved in hepatocyte function. Hepatocyte resistance to oxidative injury is mediated by ROS-dependent activation of protein kinase C, which inhibits proapoptotic signaling (38). Moreover, constitutive overexpression of CYP2E1, which is expected to cause increased ROS production, causes activation of extracellular signal-regulated kinase 1/2 with resultant protection from oxidative injury (39). Furthermore, H$_2$O$_2$ treatment of primary cultures of rat hepatocytes activates extracellular signal-regulated kinase in a dose-dependent manner and provides protection against ROS-mediated injury/cell death (40). We show that exogenous H$_2$O$_2$ can increase p85 expression, inhibit PTEN, and increase pAkt expression. Together, these data suggest that ROS signaling may be intimately involved in signal transduction, leading to steatosis in experimental NASH. The mechanism of transduction of PI 3-kinase remains to be established, although protein kinase C and extracellular signal-regulated kinase seem likely candidates as intermediates in the process given the aforementioned studies.

Intracellular H$_2$O$_2$ is potentially toxic and is rapidly degraded by catalase and other peroxidases to prevent intoxication. The balance between its production and degradation provides an opportunity for H$_2$O$_2$ to act as a signal molecule. Epidermal growth factor signal transduction in cancer cells involves generation of H$_2$O$_2$, which is involved in inhibition of protein-tyrosine phosphatase activity (41). Incorporation of excess catalase into cancer cells by electroporation completely abolishes epidermal growth factor signal transduction, showing the critical importance of the balance between production and degradation in H$_2$O$_2$-mediated signaling (41). Of interest, ROS exposure down-regulates catalase expression in renal mesangial cells by a mechanism that is dependent upon PI 3-kinase/Akt signaling through phosphorylation of the FoxO1 transcription factor (42). Further work will be needed to show if this mechanism actually accentuates H$_2$O$_2$ signaling in experimental steatosis. It is unlikely to have had an effect in these short-term experiments. More likely is that the abrupt and large excess of H$_2$O$_2$ production overwhelmed degradation, which led to increased signal.

A novel finding in the present study is that both nutrient deprivation and excess function to produce hepatocyte steatosis by the same mechanism involving ROS-dependent transduction and modulation of the PI 3-kinase pathway. Most of the effects of activating PI 3-kinase result from PIP3-dependent activation of Akt. PTEN is the natural inhibitor of Akt activation, acting by degrading PIP3. We found evidence that PTEN is functionally inactivated through phosphorylation during nutrient perturbation, which would provide amplification of the PI 3-kinase signal transduction. We also found that inhibiting the expression of PTEN in hepatocytes resulted in increased pAkt expression, providing evidence that PTEN provides a brake for PI 3-kinase signaling during normal cellular respiration. This is supported by in vivo data showing that liver-specific PTEN knock-out mice develop steatosis and liver cancer (26). These mice also have increased hepatic H$_2$O$_2$ content, which suggests that interacting with PTEN provides a natural detoxification pathway for ROS. H$_2$O$_2$ can inactivate PTEN through its reversible oxidation (21), which would consume H$_2$O$_2$ in the process. These findings together suggest that PTEN oxidation may help to regulate cellular H$_2$O$_2$ during normal respiration. Our data suggest that acute regulation of PTEN function during nutrient perturbation occurs mainly by maintaining phospho-
rulation. As with the pathway for PI 3-kinase transduction, the mechanism by which PTEN is not effectively dephosphorylated in this model is unclear. p85 itself has been shown to regulate PTEN. Liver-specific p85 knockout mice show increased hepatic PTEN expression and activity (43). Further in vitro work will be required to determine the mechanisms involved in maintaining PTEN in a relatively phosphorylated state during nutrient perturbation, and in vivo work will be required to determine if longer term dietary perturbation results in PTEN oxidation as well.

In summary, these findings support a novel pathway involving cellular metabolic stress and a patterned response leading to steatosis in this model of experimental NASH. In our conceptual model of how an abnormal diet stimulates the development of hepatic steatosis, abnormal dietary macronutrient (fuel) input or abrupt change in input to the liver results in increased generation of mitochondrial ROS, which are normally produced during aerobic energy metabolism. There is plentiful evidence that mitochondria can act as sensory organelles and send signals to the cell to adjust metabolism. In the case of a change in macronutrient input to the liver, the mitochondria “sense” the change by increasing ROS production. Our data show that an abrupt change in the ambient macronutrient composition of medium to which hepatocytes in culture are exposed results in a prompt increase in mitochondrial ROS production. This results in a signal being sent to adjust cellular metabolism to accommodate the stress. This signal is H$_2$O$_2$ produced by the action of superoxide dismutase on the burst of ROS just produced.

The PI 3-kinase-dependent cell signaling pathway has been called a “survival” pathway, because its activation promotes cell growth, activates metabolic pathways involved in cytoprotection, activates the cell cycle and promotes proliferation, and inhibits apoptosis (44, 45). H$_2$O$_2$ acts to activate and modulate the PI 3-kinase/Akt cell survival pathway, which is responsible for the many adjustments in cell metabolism needed to accommodate the stress incurred by the change in macronutrient input. The acquisition of fat (steatosis) is but one of the adjustments made. This cell survival pathway is employed as a thrust mechanism to save up fuel for a time when it is less available. Hibernating animals activate it when gorging before the winter fast (46). We believe that it is important in malfunction when people eat too much and fail to fast or otherwise use the fuel stored. The crux of this work shows the potential central role of the mitochondrial ROS/PI 3-kinase/Akt nutrient-stimulated signaling pathway in the development of steatosis. These studies provide insight into the pathogenesis of human NAFLD/NASH resulting from inappropriate nutrient intake.

Acknowledgment—We thank P. T. Schumacker, Ph.D. (Professor, Department of Pediatrics, Children’s Memorial Research Center, Northwestern University) for stimulating conversations, encouragement, and advice.

REFERENCES

1. Angulo, P. (2002) *N. Engl. J. Med.* **346**, 1221–1231
2. Brunt, E. M. (2004) *Semin. Liver Dis.* **24**, 3–20
3. Chitturi, S., and Farrell, G. C. (2001) *Semin. Liver Dis.* **21**, 27–41
4. Day, C. P. (2006) *Gastroenterology* **130**, 207–210
5. Weltman, M. D., Farrell, G. C., and Liddle, C. (1996) *Gastroenterology* **111**, 1645–1653
6. Lieber, C. S., Leo, M. A., Mak, K. M., Xu, Y., Cao, Q., Ren, C., Ponomarenko, A., and DeCarli, L. M. (2004) *Am. J. Clin. Nutr.* **79**, 502–509
7. Deng, Q. G., She, H., Cheng, J. H., French, S. W., Koop, D. R., Xiong, S., and Tsukamoto, H. (2005) *Hepatology* **42**, 905–914
8. Ahima, R. S. (2007) *Gastroenterology* **132**, 444–446
9. Koteish, A., and Diehl, A. M. (2001) *Semin. Liver Dis.* **21**, 89–104
10. Rizki, G., Arnaboldi, L., Gabrielli, B., Yan, J., Lee, G. S., Ng, R. K., Turner, S. M., Badger, T. M., Pitas, R. E., and Maher, J. J. (2006) *J. Lipid Res.* **47**, 2280–2290
11. Sahai, A., Malladi, P., Melin-Aldana, H., Green, R. M., and Whittington, P. F. (2004) *Am. J. Physiol.* **287**, G264–G273
12. Sahai, A., Pan, X., Paul, R., Malladi, P., Kohli, R., and Whittington, P. F. (2006) *Am. J. Physiol.* **291**, G55–G62
13. Feldstein, A. E., Werneburg, N. W., Canbay, A., Guicciardi, M. E., Bronk, S. F., Rydlewski, R., Burgart, L. J., and Gores, G. J. (2004) *Hepatology* **40**, 185–194
14. Browning, J. D., and Horton, J. D. (2004) *J. Clin.Invest.* **114**, 147–152
15. Perez-Carreras, M., Del Hoyo, P., Martin, M. A., Rubio, J. C., Martin, A., Castellano, G., Colina, F., Arenas, J., and Solis-Herruzo, J. A. (2003) *Hepatology* **38**, 999–1007
16. Sanyal, A. J., Campbell-Sargent, C., Mirshahi, F., Rizzo, W. B., Contos, M. J., Sterling, R. K., Luketic, V. A., Shiffman, M. L., and Clore, J. N. (2001) *Gastroenterology* **120**, 1183–1192
17. Pessayre, D., Berson, A., Fromenty, B., and Mansouri, A. (2001) *Semin. Liver Dis.* **21**, 57–69
18. Caldwell, S. H., Swerdlow, R. H., Khan, E. M., Iezzoni, J. C., Hespehende, E. E., Parks, J. K., and Parker, W. D., Jr. (1999) *J. Hepatol.* **31**, 430–434
19. Garcia-Ruiz, C., Colell, A., Morales, A., Kaplowitz, N., and Fernández-Checa, J. C. (1995) *Mol. Pharmacol.* **48**, 825–834
20. Esposito, F., Chirico, G., Montesano Gesualdi, N., Posadas, I., Ammendola, R., Russo, T., Cirino, G., and Cimino, F. (2003) *J. Biol. Chem.* **278**, 20828–20834
21. Lee, S. R., Yang, K. S., Kwon, J., Lee, C., Jeong, W., and Rhee, S. G. (2002) *J. Biol. Chem.* **277**, 20336–20342
22. Vazquez, F., and Devreotes, P. (2006) *Cell Cycle* **5**, 1523–1527
23. Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000) *Mol. Cell Biol.* **20**, 5010–5018
24. Vazquez, F., Grossman, S. R., Takahashi, Y., Rokas, M. V., Nakamura, N., and Sellers, W. R. (2001) *J. Biol. Chem.* **276**, 48627–48630
25. Cai, Z., and Semenza, G. L. (2005) *Circ. Res.* **97**, 1351–1359
26. Watanabe, S., Horie, Y., and Suzuki, A. (2005) *Hepatol. Res.* **35**, 41–46
27. Araghi-Rosati, F., Rizzuto, R., Ghibelli, L., and Cadenas, E. (2005) *Free Radic. Biol. Med.* **38**, 197–208
28. Vankoningsloo, S., De Pauw, A., Houbion, A., Tejerina, S., Demazy, C., de Longueville, F., Bertholet, V., Renard, P., Remacle, J., Hovens, M., and Blau, B. (2006) *J. Cell Sci.* **119**, 1266–1282
29. Barthel, A., and Klotz, L. O. (2005) *Biophys. Chem.* **108**, 207–216
30. Hensley, K., Robinson, K. A., Gabbita, S. P., Salsman, S., and Floyd, R. A. (2000) *Free Radic. Biol. Med.* **28**, 1456–1462
31. Benani, A., Troy, S., Carmona, M. C., Fioramonti, X., Lorsignol, A., Leloup, C., Castella, L., and Penicard, L. (2007) *Diabetes* **56**, 152–160
32. Leuop, C., Magnan, C., Benani, A., Bonnet, E., Alquier, T., Offer, G., Carriere, A., Periuet, A., Fernandez, Y., Ktorza, A., Castella, L., and Penicard, L. (2006) *Diabetes* **55**, 2084–2090
33. Du, X. L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., and Brownlee, M. (2001) *J. Clin. Invest.* **108**, 1341–1348
34. Yamagishi, S. I., Edelstein, D., Du, X. L., Kaneda, Y., Guzman, M., and Brownlee, M. (2001) *J. Biol. Chem.* **276**, 25096–25100
35. Lee, S. B., Cho, E. S., Yang, H. S., Kim, H., and Um, H. D. (2005) *Cell. Signal.* **17**, 197–204
36. Javadi, M., Beynen, A. C., Hovenier, R., Lankhorst, A., Lemmens, A. G., Terpstra, A. H., and Geelen, M. (2004) *J. Nutr. Biochem.* **15**, 680–687
37. Finkel, T. (2001) *IUBMB Life* **52**, 3–6
38. Wang, Y., Schattenberg, J. M., Rigoli, R. M., Storz, P., and Czaja, M. J.
Mitochondrial ROS Signal Steatosis via PI 3-Kinase

(2004) J. Biol. Chem. 279, 31089–31097
39. Schattenberg, J. M., Wang, Y., Singh, R., Rigoli, R. M., and Czaja, M. J. (2005) J. Biol. Chem. 280, 9887–9894
40. Rosseland, C. M., Wierod, L., Oksvold, M. P., Werner, H., Ostvold, A. C., Thoresen, G. H., Paulsen, R. E., Huitfeldt, H. S., and Skarpen, E. (2005) Hepatology 42, 200–207
41. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) J. Biol. Chem. 272, 217–221
42. Venkatesan, B., Mahimainathan, L., Das, F., Ghosh-Choudhury, N., and Ghosh Choudhury, G. (2007) J. Cell Physiol. 211, 457–467
43. Taniguchi, C. M., Tran, T. T., Kondo, T., Luo, J., Ueki, K., Cantley, L. C., and Kahn, C. R. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 12093–12097
44. Engelman, J. A., Luo, J., and Cantley, L. C. (2006) Nat. Rev. Genet. 7, 606–619
45. Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., and Waterfield, M. D. (2001) Annu. Rev. Cell Dev. Biol. 17, 615–675
46. Hoehn, K. L., Hudachek, S. F., Summers, S. A., and Florant, G. L. (2004) Am. J. Physiol. 286, R498–R504