Metabolic Regulation of Manganese Superoxide Dismutase Expression via Essential Amino Acid Deprivation

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Organisms respond to available nutrient levels by rapidly adjusting metabolic flux, in part through changes in gene expression. A consequence of adaptations in metabolic rate is the production of mitochondria-derived reactive oxygen species. Therefore, we hypothesized that nutrient sensing could regulate the synthesis of the primary defense of the cell against superoxide radicals, manganese superoxide dismutase. Our data establish a novel nutrient-sensing pathway for manganese superoxide dismutase expression mediated through essential amino acid depletion concurrent with an increase in cellular viability. Most relevantly, our results are divergent from current mechanisms governing amino acid-dependent gene regulation. This pathway requires the presence of glutamine, signaling via the tricarboxylic acid cycle/electron transport chain, an intact mitochondrial membrane potential, and the activity of both the MEK/ERK and mammalian target of rapamycin kinases. Our results provide evidence for convergence of metabolic cues with nutrient control of antioxidant gene regulation, revealing a potential signaling strategy that impacts free radical-mediated mutations with implications in cancer and aging.

Nutrient availability relative to both carbohydrates and amino acids (AAs) in the mammalian diet, has potentially critical impacts on metabolic flux and ultimately the generation of ATP and its equivalents. With constantly changing constituents associated with the mammalian diet, organisms have adapted metabolic strategies to efficiently accommodate changes in the availability of critical nutrients. Extensive studies have addressed the importance of glucose excess (1) and deprivation of essential AA has been demonstrated to evoke responses at both the transcriptional and post-transcriptional levels for genes such as asparagine synthetase (ASNS), CCAAT/enhancer-binding protein homologous protein, cationic AA transporter (Cat-1), sodium-coupled neutral AA transporter system A (SNAT2), and insulin-like growth factor-binding protein-1 (IGFBP-1) (3). Fernandez et al. (10) have also demonstrated the existence of an internal ribosome entry site within the 5′-untranslated region of the cat-1 gene that controls translation of this transport protein under conditions of AA depletion.

Tissue and cellular adaptation to nutrient availability also affects carbon and nitrogen utilization through glycolysis, the tricarboxylic acid cycle, and ultimately the aerobic generation of ATP via electron transport. A critical consequence of nutrient availability and subsequent metabolism is the generation of reactive oxygen species (ROS) as by-products of normal metabolism (11). Previous estimates have indicated that under normal aerobic and nutrient conditions, 0.1% of consumed oxygen is released as superoxide radicals from mitochondrial electron transport (12). It has also been established that a significant increase in the levels of ROS occurs under pathological conditions involving the synthesis and action of many pro-inflammatory mediators (13, 14). Furthermore, the connection between nutrient levels and the generation of ROS is underscored when considering that caloric restriction can significantly delay the aging process (15), which may be explained by a reduction in metabolic flux and a concomitant decline in ROS production (16). This observation is also consistent with the mitochondrial theory of aging (17), which implicates continuous generation of ROS as a critical factor for damage to mitochondrial DNA as well as oxidative reactions with components of the cytosol and nucleus.

Mutations in genes from the insulin-like signaling network in Caenorhabditis elegans, such as age-1, a homologue of the mammalian phosphatidylinositol-3-OH kinase, and daf-2, a homologue of the insulin or insulin-like growth factor receptor family, cause a life span extension phenotype, with animals living twice as long as the wild type (18). These mutations may

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2 The abbreviations used are: AA, amino acid; MnSOD, manganese superoxide dismutase; mTOR, mammalian target of rapamycin; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; ROS, reactive oxygen species; RT, reverse transcription; MEM, minimal essential medium; EBSS, Earle's balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FBS, fetal bovine serum; siRNA, short interfering RNA; hGH, human growth hormone; IL, interleukin; PBS, phosphate-buffered saline; ASNS, asparagine synthetase; 2-DOG, 2-deoxy-D-glucose; 3-NPA, 3-nitropropionic acid; KMV, α-keto-β-methyl-n-valeric acid.

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propagate their effects by conferring resistance to oxidative stress, possibly through increases in the antioxidant enzyme, manganese superoxide dismutase (MnSOD) (18). Studies in yeast (19) and Drosophila (20, 21) have also implied overexpression of MnSOD with an increase in organism life span. Furthermore, Kokoszka et al. (22) have demonstrated that MnSOD heterozygous mice displayed increased mitochondrial dysfunction marked by increased proton leakage, inhibition of respiration, and the accumulation of mitochondrial oxidative damage. These studies also provided a link between chronic oxidative stress in the heterozygous mice and a premature induction of apoptosis, thus implicating the importance of MnSOD in cell death and aging. These observations are also consistent with data demonstrating the potent anti-apoptotic activity associated with overexpression of this mitochondrial localized antioxidant enzyme in a variety of mitochondria-dependent cell death pathways (23–26). Additionally, the significant induction of MnSOD gene expression by pro-inflammatory cytokines (13, 27, 28) is also in line with a cell survival strategy that accompanies increased levels of ROS associated with the inflammatory response.

Therefore, we postulated that nutrient availability, in the form of either glucose or AA deprivation, may have relevant metabolic and cell survival benefits through elevation of MnSOD levels. Our results establish a nutrient-dependent regulation pathway for MnSOD that is novel when compared with the cellular mechanisms that are currently proposed to orchestrate the induction of gene expression via essential AA deprivation (3). We have demonstrated that, in contrast to all previously studied AA-regulated genes, the induction of MnSOD by histidine depletion, a representative essential AA, requires the presence of millimolar levels of glutamine (Gln). We have shown that this regulatory pathway requires intracellular signals through the tricarboxylic acid cycle and electron transport chain with a dependence on mitochondrial membrane potential but not ATP levels. Our results also demonstrate that this unique AA-dependent regulatory pathway necessitates normal signaling through both the MEK/ERK and mTOR pathways.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—2-Methoxyestradiol, KMV, 3-nitropropionic acid, malonate, actinomycin-b, cycloheximide, antimycin, oligomycin, and 2,4-dinitrophenol were obtained from Sigma. SB202190, SB203580, PD98059, JNK (c-Jun N-terminal kinase) inhibitor II, and U0126 were purchased from Calbiochem. Fluorocetate and rapamycin were obtained from Fluka, St. Louis, and LC Laboratories, Woburn, MA, respectively. Phospho-p70 S6 kinase (Thr-389), phospho-4E-BP1, and phospho-p70 S6 kinase (Thr-421/Ser-424) antibodies were purchased from Cell Signaling, Danvers, MA, and MnSOD was from StressGen, San Diego.

**Cell Culture and Treatment Conditions**—HepG2 (human hepatoma) cells were maintained in minimal essential medium (MEM) (Sigma), pH 7.4, supplemented with 25 mM NaHCO3, 4 mM Gln, antibiotic/antimycotic (Invitrogen), and 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C with 5% CO2. All data involve the use of this cell line. Cells were grown to 50–65% confluency on 10-cm dishes and split 1:8 into 60-mm dishes for 24 h. In all experiments, medium was changed 12 h before the start of the experiment to ensure the AA levels in general are not depleted. The cells were washed three times with phosphate-buffered saline (PBS), incubated in 3 ml of the appropriate medium, and collected at the indicated times for either Northern or immunoblot analysis. All experiments were performed with 10% dialyzed FBS.

For experiments where AA other than histidine are depleted, Earle’s Balanced Salt Solution (EBSS) (Sigma) was used and supplemented with antibiotic/antimycotic, a vitamin mixture (Invitrogen), and 10% dialyzed FBS. Each AA was then added individually to the medium at the same levels as in the MEM while omitting the AA being tested. For the “AA add back” experiments, the same EBSS base and supplements were used, and then only the indicated AA was added at a concentration of 5 mM. The pH of the medium was maintained at 7.2–7.4 and adjusted (when necessary) with 0.1 N NaOH or HCl. Stock solutions of inhibitors were adjusted to a neutral pH before treatments.

**RNA Isolation and Northern Analysis**—Total RNA was isolated from cells as described by the Chomczynski and Sacchi method with modifications (28). Five to 10 μg of total RNA were fractionated on 1% agarose, 6% formaldehyde gels, electrotransferred to a Zeta-Probe membrane (Bio-Rad), and UV cross-linked. Membranes were then incubated for 1 h in a prehybridization buffer and incubated with a 32P-radiolabeled gene-specific probe generated by random primer extension overnight at 62 °C. Membranes were then washed with a high stringency buffer at 66 °C and exposed to x-ray film.

**Generation of cDNA**—To generate cDNA for real time PCR analysis SuperScriptTM first strand synthesis kit from Invitrogen was used. 1 μg of total RNA isolated as described was used as the template. Prior to further analysis, double distilled H2O was added to a final reaction volume of 100 μl.

**Real Time RT-PCR**—2 μl of cDNA generated from first strand synthesis (as described above) was used as the template for real time PCR. To this, 0.3 μM of each primer was added, 12.5 μl of iTaq™ SYBR® Green Supermix with ROX (Bio-Rad), and water to a final volume of 25 μl. The Applied Biosystems 7000 sequence detection system was used with the following parameters: cycle 1 (95 °C for 10 min) 1 time; cycle 2 (95 °C for 15 s, 60 °C for 1 min) for 40 cycles. The ΔΔCT method was used to determine the relative fold changes, normalized to the cyclophilin A gene, and is described in Ref. 29. Primers for cyclophilin A are as follows: forward, 5′-CAT CCT AAA-3′ and reverse, 5′-GCT GGT CTT GCC ATT CCT G-3′.

**De Novo Transcriptional Analysis of MnSOD**—RNA was isolated as described previously with the addition of DNase I treatment. Heterogeneous nuclear RNA levels were then quantified by real time RT-PCR.

**Growth Hormone Reporter Constructs**—Regions of the MnSOD gene were cloned into a human growth hormone (hGH) reporter plasmid (30). A phage artificial chromosome clone, obtained from the Sanger Institute (RP1-56L9), containing the entire human MnSOD gene was used to clone regions of interest into a promoterless growth hormone reporter plasmid (pOGH). A 3.6-kb BamHI fragment of the human promoter
was digested from the phage artificial chromosome vector and separated on a 0.7% agarose gel, gel-purified, and cloned into the BamHI site of pØGH. Unique restriction enzyme sites were used to generate 1.4-, 1.3-, 1.1-, and 0.83-kb promoter fragments. To generate the promoter constructs in conjunction with the human MnSOD enhancer, a 488-bp fragment was digested from a previously generated construct (31). The 488-bp human enhancer fragment was subcloned into the HindIII site of the hGH constructs containing the indicated human MnSOD promoter. A plasmid containing a minimal viral thymidine kinase promoter coupled to the pØGH plasmid was used with the human MnSOD enhancer fragment as described previously (31).

Transient Transfection of Reporter Constructs—HepG2 cells were cultured as described previously and transfected at ~50% confluence in a 10-cm dish. The reporter plasmid containing different regions of the MnSOD gene were transiently transfected using a FuGENE 6 transfection reagent (Roche Applied Science). For each 10-cm dish, 15 μl of the FuGENE 6 transfection reagent was diluted to a final volume of 600 μl in serum-free MEM. 5 μg of reporter plasmid was then added, and the reaction was incubated at room temperature for 30 min and then transferred to HepG2 cells. After 24 h, the cells were split 1:10 into 35-mm dishes and incubated for another 12 h after which the cells were then incubated in either FED or His media. pØGH was also transfected to ensure that the transfection itself or the hGH plasmid did not have an effect on the MnSOD message. Northern analyses were used to evaluate the effect of histidine deprivation on the MnSOD message. A fragment from the hGH cDNA was used to generate a radioabeled probe for Northern analysis.

Protein Isolation and Immunoblot Analysis—HepG2 cells were washed twice with ice-cold PBS and lysed with a buffer containing both protease (Roche Applied Science) and phosphatase inhibitors (Calbiochem), in 1M Tris, pH 7.5, 5M NaCl, containing both protease (Roche Applied Science) and phosphatase inhibitors (Calbiochem), and 0.5 EDTA pH 8, and Triton X-100 (TBST). Protein concentrations were determined by bicinchoninic acid (BCA) assay (Pierce). 10–20 μg of total cellular protein was separated on a Tris–HCl, SDS–polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane (Amer sham Biosciences). The membranes were then blocked for 1 h with 5% nonfat dry milk in TBST at room temperature. The membranes were incubated at 4°C with primary antibody overnight, washed three times with TBST, incubated with a secondary for 2 h, washed again three times, and subjected to ECL chemiluminescence (Amer sham Biosciences).

Cell Viability—Cell viability was determined using a 3-(4,5-di methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Invitrogen). 10 μl of 12 mM MTT dissolved in PBS was added to the culture medium and allowed to incorporate into the cells for 4 h at 37°C. The media were then removed and the cells rinsed with PBS. Cells were solubilized in Me2SO diluted in PBS and incubated for 10 min at 37°C. The cells were then mixed and the plates read at 540 nm in a Spectra-max plus 384 plate reader.

siRNA—HepG2 cells were transfected with a final concentration of 100 nM SMARTpool® MnSOD siRNA (Dharmacon) using DharmaFECT™ 4 siRNA transfection reagent (Dharmacon). A cyclophilin-specific siRNA (Dharmacon) was employed as a control for off-target effects of siRNA.

ATP Measurements—ATP levels were measured from HepG2 cells utilizing the ATP bioluminescent kit from Sigma. After 12 h of treatment cells were trypsinized; 3 ml of medium was added, the cells were mixed thoroughly, and 1 ml was transferred to a 1.5-ml microcentrifuge tube. A 100-μl aliquot of ATP assay solution (diluted 1:25) was added to a fresh tube and incubated at room temperature for 3 min, allowing for endogenous ATP to be hydrolyzed and decreasing the background. In a separate 1.5-ml microcentrifuge tube, the reaction was incubated at room temperature for 30 min and then transferred to HepG2 cells. After 24 h, the cells were split 1:10 into 35-mm dishes and incubated for another 12 h after which the cells were then incubated in either FED or His media. pØGH was also transfected to ensure that the transfection itself or the hGH plasmid did not have an effect on the MnSOD message. Northern analyses were used to evaluate the effect of histidine deprivation on the MnSOD message. A fragment from the hGH cDNA was used to generate a radioabeled probe for Northern analysis.

RESULTS AND DISCUSSION

Nutrient availability in the mammalian diet has a potentially critical impact on metabolic flux, the generation of ATP, and as a consequence, the generation of mitochondrially derived ROS. Given the role of MnSOD in the detoxification of mitochondrially derived ROS, we postulated that nutrient availability might have direct effects on the levels of MnSOD gene expression. To test this hypothesis, the response of MnSOD in cell culture to the exclusion of a single essential AA, histidine, was evaluated by Northern analysis in the human hepatoma cell line, HepG2, and compared with the documented response to pro-inflammatory mediators. It has been previously established that lipopolysaccharide, interleukins 1 and 6 (IL-1β and IL-6), tumor necrosis factor-α, and interferon-γ induce MnSOD at the mRNA and protein levels (27, 32–35).

The top panel of Fig. 1A shows MnSOD mRNA levels in HepG2 cells incubated for 12 h in complete MEM medium (FED), MEM lacking histidine (−His), or treated with either lipopolysaccharide, tumor necrosis factor-α, IL-1β, or IL-6. Two mRNA species are produced from the human MnSOD gene because of alternative polyadenylation (36, 37). Steady state MnSOD mRNA levels are induced in response to −His, relative to FED, and addition of 5 mM histidine to FED conditions (1st lane of Fig. 1A (+His)) further reduced basal levels, demonstrating the depletion of histidine over the 12-h incubation time and subsequent increases in MnSOD mRNA levels (zero and FED). This observation could have profound effects because most conventional cell culture procedures do not recommend feeding of cells for up to 48 h. To ensure that all experiments have similar MnSOD basal levels, fresh medium was given to the cells 12 h before the start of each experiment. Because HepG2 cells preferentially produce the 4-kb message
in response to −His, this is the species referred to and utilized in densitometry for all experiments. In all Northern analyses, the large ribosomal subunit 7a (L7a) was used as an internal loading control.

The immunoblot analysis at the bottom of Fig. 1A also demonstrates associated increases of MnSOD protein by 48 h. This induction is also reproducible in another human hepatoma cell line, HuH7, as well as, less robustly, in human lung fibroblast and epithelial cells (data not shown).

Many other genes known to be regulated by AA deprivation also respond to glucose starvation (3). In addition, glucose metabolism may have profound downstream effects on the production of mitochondrially derived ROS. To evaluate the specificity of changes in MnSOD mRNA levels, cells were starved for glucose (−Glc) and compared with −His. As shown in Fig. 1B, MnSOD mRNA levels are induced only in response to AA deprivation. As a positive control for glucose deprivation, the membranes were reprobed for glucose-regulated protein (GRP78) (38). Fig. 1C is a summary of densitometric data from three independent experiments. Two other known inducers of the endoplasmic reticulum stress response (39), thapsigargin and tunicamycin, were also tested and, similar to glucose deprivation, showed no induction of MnSOD mRNA (data not shown).

To further characterize the MnSOD gene induction in response to AA deprivation, mRNA levels were evaluated when each individual AA was omitted from complete medium and incubated for 2, 6, and 12 h. As shown in the representative examples in Fig. 1D, depletion of essential AA other than His also causes a similar increase in MnSOD mRNA levels, with the exception of tryptophan. On the other hand, depletion of nonessential AA from culture medium had no effect. A densitometric and statistical summary is shown in Fig. 1E.

Given the uniqueness of the induction of MnSOD by essential amino acid starvation (−His condition), we next evaluated a potential physiological role for MnSOD during amino acid deprivation. Cell viability was measured using an MTT assay. As illustrated in Fig. 2A, incubation in −His medium reproducibly causes an increase in cell viability (∼48%) as compared with the FED condition. To evaluate the role of MnSOD in the increased cell survival, cells were also treated with 2-methoxyestradiol, which has been shown to inhibit MnSOD (40, 41). These data (Fig. 2A) also demonstrate that 10 μM 2-methoxyestradiol eliminates the increased cell survival associated with the −His conditions, whereas the inhibitor had no effect on cells grown in the FED condition.

To determine whether −His conditions offer a cytoprotective advantage during stress conditions, cells were preincubated for 24 h in −His medium followed by exposure to sublethal levels of UV light. The cell survival curves in Fig. 2B...
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A. **FIGURE 2.** A, bar graph representing cell viability as determined by MTT assay at 540 nm. HepG2 cells were incubated in either FED or −His medium with the indicated amount of 2-methoxyestradiol (2-ME) (μM) for 24 h. B, line graph representative of HepG2 cells exposed to increasing duration of broad spectrum UV light cultured in either FED or −His medium. 24 h following UV exposure, cell survival was determined by the MTT assay and represented as percent cell death. C, immunoblot analysis of MnSOD from HepG2 cells transfected with siRNAs for 3 and 5 days. D, bar graph of percent cell death of HepG2 cells transfected with the indicated siRNA after incubation in FED or −His media for 24 h with a 1-min UV treatment. MTT assays were performed 24 h after UV treatment, and results are represented as percent cell death. Data points in A, B, and D are the means ± S.E. (n = 3). An asterisk denotes significance as compared with the FED condition in A and B or FED with nonspecific siRNA in D as determined by a Student’s t test to a value of p = 0.05. A plus sign in D denotes p = 0.05 as compared with FED with MnSOD siRNA.

***Figure 2***

- **A.** Bar graph representing cell viability as determined by MTT assay at 540 nm. HepG2 cells were incubated in either FED or −His medium with the indicated amount of 2-methoxyestradiol (2-ME) (μM) for 24 h. **B.** Line graph representing HepG2 cells exposed to increasing duration of broad spectrum UV light cultured in either FED or −His medium. 24 h following UV exposure, cell survival was determined by the MTT assay and represented as percent cell death. **C.** Immunoblot analysis of MnSOD from HepG2 cells transfected with siRNAs for 3 and 5 days. **D.** Bar graph of percent cell death of HepG2 cells transfected with the indicated siRNA after incubation in FED or −His media for 24 h with a 1-min UV treatment. MTT assays were performed 24 h after UV treatment, and results are represented as percent cell death. Data points in A, B, and D are the means ± S.E. (n = 3). An asterisk denotes significance as compared with the FED condition in A and B or FED with nonspecific siRNA in D as determined by a Student’s t test to a value of p = 0.05. A plus sign in D denotes p = 0.05 as compared with FED with MnSOD siRNA.

Demonstrate that −His conditions can reduce cell death following UV exposure. The level of cytoprotection was most significant at 1 and 2 min of UV exposure with an ~50 and 70% reduction in cell death at these time points, respectively. To further address the specific role of MnSOD in the increase of cell survival associated with depletion of essential AA, we exposed cells to an siRNA specific to MnSOD as compared with a nonspecific siRNA. To ensure that MnSOD protein levels were sufficiently reduced, HepG2 cells were transfected with siRNAs and protein collected on days 3 and 5 for immunoblot analysis. Fig. 2C demonstrates that the specific siRNA can significantly reduce endogenous MnSOD protein levels. HepG2 cells were transfected with specific and nonspecific siRNA and then incubated in FED or −His medium for 24 h. Each group was then exposed to UV radiation for 1 min and then assayed for cell viability. Fig. 2D illustrates that MnSOD siRNA can cause an ~25% increase in cell death in FED cells. Analogous to the results in Fig. 2B at 1 min of UV exposure, treatment of cells grown in −His conditions with a nonspecific siRNA also illustrates the cytoprotective effect of −His conditions. Alternatively, cells grown in −His conditions and transfected with MnSOD siRNA showed an ~74% or ~6-fold increase in cell death relative to the FED or −His cells transfected with nonspecific siRNA, respectively. These data demonstrate that induction of MnSOD by essential amino acid deprivation offers a distinct survival advantage under both normal and stress conditions. These results may also have implications in nutritional studies regarding increase in life span associated with dietary restrictions.

Given that deprivation of a single AA resulted in the induction of MnSOD mRNA, we tested if total AA deprivation would result in a similar effect. Notably, MnSOD mRNA levels were not elevated in HepG2 cells incubated in medium lacking all AA (EBSS) (Fig. 3A). This is contrary to the response detected for other genes regulated by depletion of essential AA, in that these genes can respond to single or complete AA deprivation (42, 43). In fact, we know of no other gene regulated by AA deprivation that is not induced in response to complete AA depletion. To address the AA specificity in an “add back” experiment, cells were incubated in EBSS or in EBSS with the addition of a single AA. We analyzed the effect of each of the amino acids; a subset of the data is shown in Fig. 3A. Our findings indicate that Gln is the only AA that, when added back to EBSS, is able to re-establish the induction of MnSOD mRNA levels. To further address the specificity of this response, we also evaluated asparagine synthetase (ASNS) (3, 42, 43), a gene known to be induced by both single and complete AA deprivation (Fig. 3A, 3B, and 4). These data demonstrate MnSOD is clearly not regulated in a manner identical to ASNS, and moreover, the addition of Gln to EBSS is sufficient to re-establish the induction of MnSOD with no effects on ASNS. Fig. 3A, 3B, and 42 shows a separate experiment directly comparing MnSOD and ASNS levels in −His, EBSS, and EBSS +Gln as well as a quantitative summary of Gln add back as compared with MnSOD mRNA levels in EBSS at 12 h.
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To determine the relevance of Gln to the induction of MnSOD mRNA by AA deprivation, we incubated HepG2 cells in −His or in −His/−Gln. Fig. 3C demonstrates that the absence of Gln completely abolishes the −His induction (−His/−Gln). Given the potential use of glutamine and/or glucose as a carbon/fuel source, we also evaluated the effects of glucose depletion in combination with −His. Contrary to the effects of −Gln, glucose (−Glc) deprivation did not alter the −His response (Fig. 3C, right panel).

We next evaluated HepG2 cells in culture medium lacking histidine in conjunction with depletion of other amino acids (Fig. 3D, left panel); neither tryptophan nor methionine removal blocked the induction in response to histidine deprivation, further demonstrating the importance of Gln to the induction of MnSOD. Another component of complete medium which could be, in part, responsible for the increases in MnSOD mRNA levels is the presence of vitamin supplements (Fig. 3D, right panel). These data illustrate that vitamins are not necessary for the observed induction and in an independent experiment that no serum dependence was observed (data not shown). To determine the levels of Gln required for the induction of MnSOD, we incubated cells in either −His or EBSS with increasing amounts of Gln (Fig. 3E). As shown, 1 mm Gln is adequate to re-establish MnSOD mRNA induction in −His or EBSS, with no effect on FED cells.

To establish a mechanistic link between Gln and MnSOD gene activation, we hypothesized that Gln metabolism could be connected to increases in MnSOD mRNA levels. The primary pathway for utilization of Gln is catabolism through the tricarboxylic acid cycle. Gln enters the tricarboxylic acid cycle through glutamate and α-ketoglutarate, so we investigated the contribution of the tricarboxylic acid cycle to the induction of MnSOD by utilizing selective inhibitors to several different key enzymes. We first targeted the inhibition of aconitase with 3-fluoroacetate (44, 45), which occurs through the intracellular conversion to the aconitase substrate inhibitor, fluorocitrate. As shown in Fig. 4A, 3-fluoroacetate inhibited the induction of MnSOD mRNA levels by −His, in a concentration-dependent manner, with no effect on ASNS. A quantitative summary of the data is shown in Fig. 4B.

We next targeted the entry point for Gln into the tricarboxylic acid cycle, α-ketoglutarate dehydrogenase. α-Ketoglutarate is derived from two main sources, either isocitrate, through a decarboxylation reaction, or from glutamate through the deamination of Gln. To target this we used KMV, a structural analogue of α-ketoglutarate and a competitive inhibitor of the α-ketoglutarate dehydrogenase complex (46). The results shown in Fig. 4C demonstrate that, similar to fluoroacetate, KMV causes a significant inhibition of the −His induction of MnSOD, again with no effect on ASNS expression.

In light of the functional anti-oxidant role played by MnSOD in mitochondria, we next evaluated the succinate dehydrogenase complex, a component of both the tricarboxylic acid cycle and electron transport chain (complex II) that resides in the inner membrane and directly transfers electrons to ubiquinone, bypassing the first phosphorylation site in electron transfer. We chose
two inhibitors of the succinate dehydrogenase complex, 3-nitropropionic acid (3-NPA), a covalent and irreversible inhibitor (47), and malonate, a competitive inhibitor (48, 49). As shown in Fig. 4D, 3-NPA caused a significant inhibition of the −His induction, with no response to treatment with increasing concentrations of malonate (Fig. 4E). This is consistent with the biochemical argument that, as a competitive inhibitor, malonate can be displaced when cellular succinate concentrations are elevated, presumably the case when adequate levels of Gln are available. Interestingly, 3-NPA did cause the inhibition of the ASNS induction by −His, whereas fluoroacetate and KMV had no effect (data not shown).

Given the importance of the tricarboxylic acid cycle in MnSOD gene activation by AA deprivation and the role of this cycle in the generation of reducing units for consumption through oxidative phosphorylation, we next evaluated the importance of the electron transport chain to this induction. Of particular note is that superoxide radicals, the substrates for MnSOD enzymatic activity, are formed as by-products at both complexes I and III of the electron transport chain.

We first tested an inhibitor of complex III, antimycin A, which we have previously demonstrated inhibits the induction of MnSOD by tumor necrosis factor−α, but not the induction by IL-1β or that lipopolysaccharide (14). As shown in Fig. 5A, exposure to increasing concentrations of antimycin A completely inhibited −His induction of MnSOD with effective concentrations in the low nanomolar range. The F$_{1}$F$_{0}$-ATP synthase complex utilizes the proton gradient across the intermembrane space to drive ATP synthesis. We next evaluated the importance of the electrochemical gradient in the generation of ATP through an inhibitor of the F$_{1}$F$_{0}$-ATP synthase complex, oligomycin (14, 50, 51). The results shown in Fig. 5B demonstrate the inhibition of MnSOD induction in the low nanomolar range of oligomycin, implying that the underlying signaling mechanism either requires adequate ATP levels or an intact electrochemical gradient.

With the metabolic perturbations potentially associated with alterations in nutrient levels, we measured the levels of ATP under each of our growth conditions. To first establish a growth condition that would definitively reduce cellular ATP levels as a positive control, we treated cells with 2-deoxy-D-glucose (2-DOG), a non-metabolizable form of glucose, creating a cellular state analogous to glucose depletion (52). As previously demonstrated in Fig. 1B, conditions of limiting glucose did not induce MnSOD levels nor affect the induction by −His (Fig. 3C). The data presented in Fig. 5C confirms that 2-DOG does not affect MnSOD levels in FED, −His, or EBSS −/+Gln. ATP levels were then measured under a variety of experimental conditions, as shown in Fig. 5D. Comparable reductions in ATP levels were observed for all conditions relative to complete medium (FED). These data indicate that the induction of MnSOD levels by −His conditions is not dependent on alterations in ATP levels because 2-DOG and −His both result in reduced ATP levels, yet the former condition (Fig. 5C) has no effect on MnSOD mRNA levels. In addition, these results also demonstrate that the −His
condition only reduces ATP levels by ~25% as compared with reductions of >=50% in either EBSS or the FED condition with 2-DOG. Of equal importance is that the re-addition of Gln to EBSS results in a recovery of ATP levels.

The driving electromotive force for ATP generation and an indicator for the overall competence and healthiness of mitochondria is the existence of an intact proton gradient. Therefore, we evaluated an uncoupler of electron transport and oxidative phosphorylation, 2,4-dinitrophenol (53). In the mitochondrial intermembrane space, 2,4-dinitrophenol binds free protons transporting them into the mitochondrial matrix and, in so doing, bypassing the F$_{1}$F$_{0}$-ATPase production of ATP and effectively uncoupling electron transport. As shown in Fig. 5E, increasing concentrations of 2,4-dinitrophenol inhibited the induction of MnSOD mRNA levels by −His, strongly implicating the importance of an intact proton gradient.

increase in the phosphorylation of ERK1/2 and that inhibition of the ERK signal transduction pathway causes a reduction in the −His-dependent increase in p21 mRNA (56). With the unique nature of the MnSOD induction, relative to other genes such as ASNS, we also tested other members of the MAPK and JNK pathways (Fig. 6, A and B). Inhibitors for p38 MAPK and JNK had no effect on the induction by either −His or EBSS + Gln (Fig. 6A). On the other hand, inhibitors targeting ERK1/2 activity, PD98059 and U0126, blocked both the −His and EBSS + Gln inductions (Fig. 6B with densitometric analysis in Fig. 6C).

Similarly, another signaling pathway, also dependent on AA availability, that plays a central role in monitoring and regulating downstream translational events is the mTOR (4–6, 57–61). We treated HepG2 cells with rapamycin, a potent inhibitor of mTOR (Fig. 7A). Rapamycin treatment caused a dose-dependent inhibition of the MnSOD induction in −His.

The classic cellular sensing mechanism for detecting essential AA deprivation, referred to as the amino acid response pathway, is associated with an increase in the concentration of uncharged tRNA leading to the activation of the GCN2 kinase (54). The activation of GCN2 kinase leads to phosphorylation of translation initiation factor eIF-2α, causing a decline in global protein synthesis (54). The GCN2-dependent pathway can be activated by either deprivation of a single essential AA or complete AA deprivation (EBSS) (3). As we have established, the induction of MnSOD occurs only by depletion of a single essential AA and not by complete AA deprivation, implying that the GCN2 pathway may not be utilized as the sensor for MnSOD induction. A further distinction is the dependence on Gln for MnSOD induction which has not been established as a requirement for the GCN2 pathway.

We therefore investigated other relevant signaling pathways potentially linking the induction of MnSOD to either −His or EBSS + Gln. Franchi-Gazzola et al. (55) have previously demonstrated that the MAPK pathway, through an increase in ERK1/2 phosphorylation and activity, was involved in the induction of fibroblast system A transport activity following incubation in AA-free medium. We have more recently demonstrated that histidine deprivation causes an increase in the phosphorylation of ERK1/2 and that inhibition of the ERK signal transduction pathway causes a reduction in the −His-dependent increase in p21 mRNA (56). With the unique nature of the MnSOD induction, relative to other genes such as ASNS, we also tested other members of the MAPK and JNK pathways (Fig. 6, A and B). Inhibitors for p38 MAPK and JNK had no effect on the induction by either −His or EBSS + Gln (Fig. 6A). On the other hand, inhibitors targeting ERK1/2 activity, PD98059 and U0126, blocked both the −His and EBSS + Gln inductions (Fig. 6B with densitometric analysis in Fig. 6C).

Similarly, another signaling pathway, also dependent on AA availability, that plays a central role in monitoring and regulating downstream translational events is the mTOR (4–6, 57–61). We treated HepG2 cells with rapamycin, a potent inhibitor of mTOR (Fig. 7A). Rapamycin treatment caused a dose-dependent inhibition of the MnSOD induction in −His.
conditions, which strongly implies that induction of MnSOD by essential amino acids requires normal mTOR activity for the induction to occur. Interestingly, rapamycin had no effect on the induction of MnSOD mRNA levels when cells were incubated in EBSS/H11001Gln conditions. Fig. 7B illustrates a quantitative summary of these data. The ability of rapamycin to distinguish between the effects of −His and EBSS + Gln conditions is in contrast to the ability of ERK1/2 inhibitors to block both modes of MnSOD induction. Furthermore, the ability of rapamycin to distinguish between the two conditions strongly suggests that, although necessary for the induction by −His, the addition of Gln to EBSS may constitute a distinct but interdependent pathway for induction of MnSOD. To further distinguish the importance of mTOR activity in −His versus EBSS + Gln conditions, we examined one of the critical downstream effectors of mTOR activity, p70 S6K (62, 63). Fig. 7C illustrates a p70 S6K immunoblot for cells in a variety of growth conditions in conjunction with exposure to rapamycin. The FED condition demonstrates mTOR-dependent phosphorylation of threonine 389 (Thr-389) in p70 S6K, a relevant mTOR target (64). The antibody for p70 S6K also recognizes an 85-kDa isoform of the p70 S6 kinase protein, and the arrow marks the 85-kDa isofrm of the p70S6 kinase protein, and the arrow marks the p70 S6K phosphorylated at Thr-389.

Amino acid control of gene expression has been described at many levels, including transcription, translation, and mRNA stability (3, 42). To help elucidate the mechanism of increased MnSOD mRNA levels, in response to amino acid deprivation, we measured heterogeneous nuclear RNA levels for MnSOD (66). This involves the assessment of the intron-containing precursor of mRNA by real time RT-PCR as a direct evaluation of the level of de novo transcription. Fig. 8A demonstrates that
MnSOD heterogeneous nuclear RNA is increased in both −His and EBSS + Gln conditions, demonstrating that these inductions require de novo gene transcription, analogous to induction of ASNS by −His (67).

To identify potential DNA regulatory elements involved in MnSOD gene induction in response to essential amino acid deprivation, an hGH reporter plasmid was coupled to MnSOD promoter constructs. An illustration of the MnSOD genomic structure indicating the cytokine-responsive enhancer and the promoter fragments used is provided in Fig. 8B. As a control, the transfected cells were also exposed to another established inducer of MnSOD, IL-1β (28). As has been demonstrated previously, IL-1β induction does not occur through the promoter alone but requires an intronic cytokine-responsive enhancer element (31). Given that the enhancer element is known to regulate MnSOD induction by cytokines, this region, in conjunction with each of the illustrated promoter fragments, was also evaluated following histidine deprivation (Fig. 8C). The results from these constructs demonstrate a small, but reproducible, induction with the −3.4- and −1.4-kb constructs with the loss of −His induction associated with the −0.83-kb promoter deletion. As expected, the enhancer element conferred IL-1β-dependent induction.

To determine the contribution of the enhancer fragment to the induction of MnSOD by amino acid deprivation versus the overall transcription levels of the reporter plasmid, the enhancer was placed in front of a heterologous viral thymidine kinase (TK) promoter. As demonstrated in Fig. 8D, the enhancer does not respond to −His conditions in the absence of the human MnSOD promoter. As shown previously, the human enhancer alone in conjunction with the thymidine kinase promoter does respond to IL-1β (31). These results point to the importance of the region between 1.4 and 0.83 kb upstream of the transcriptional initiation site.

A recent study by Kops et al. (68) has demonstrated the importance of a forkhead box, sub-group O (FOXO) regulatory element, located between 1.3 and 1.1 kb upstream of the transcriptional initiation site of the human MnSOD gene. To test the role of the FOXO element in the induction by amino acid deprivation, we deleted the canonical FOXO DNA-binding site from the 1.3-kb promoter fragment. Northern blots were performed using promoter constructs containing 3.4, 1.4, 1.3, 1.1, and 0.83 kb constructs as well as one construct containing the 1.3-kb promoter from which the FOXO-binding site has been deleted. These

**FIGURE 8.** A, real time PCR analysis of heterogeneous RNA levels of MnSOD performed as described under “Experimental Procedures.” B, illustration of the MnSOD genomic structure indicating the cytokine-responsive enhancer and the fragments derived from the proximal promoter. C, representative Northern analysis of the 3.4-, 1.4- and 0.83-kb promoter fragments with the cytokine-responsive intronic enhancer in the hGH vector. IL-1β-treated cells are included to demonstrate the functional activity of the enhancer element. D, representative Northern analysis of the MnSOD intronic enhancer coupled to the heterologous thymidine kinase (TK) promoter in the hGH reporter in FED, −His, and IL-1β-treated cells. E, densitometric data from three independent Northern analyses of the indicated hGH reporter constructs as shown in B. The 1.3-kb ΔFOXO bar represents a construct where the putative FOXO-binding site has been deleted. Data points are the means ± S.E. (n = 3). The asterisks in A and E denote significance as compared with the FED condition as determined by a Student’s t test to a value of p = 0.05.
data, summarized by densitometry in Fig. 8E, demonstrate a role for the FOXO DNA-binding site in the regulation of MnSOD by histidine deprivation.

In summary, organisms have adopted metabolic strategies to accommodate changes in the availability of critical nutrients. Recent studies have addressed the following: 1) the importance of glucose excess, 2) deprivation, and 3) AA availability on metabolic and nuclear events. A critical consequence of nutrient availability and subsequent metabolism is the generation of ROS (69, 70). Furthermore, the connection between nutrient levels and the generation of ROS is underscored when considering that caloric restriction can significantly delay the aging process (15), an observation in line with the free radical theory of aging (16, 17). One contributing factor is the importance of the mitochondrial localized anti-oxidant enzyme, MnSOD, to energy/redox metabolism, aging, and disease pathologies (71). Our results demonstrate that depletion of individual essential AA, but not glucose, causes induction of human MnSOD mRNA and protein levels. Contrary to effects on other AA-regulated genes, such as ASNS (3, 72), which are regulated by mRNA and protein levels. Contrary to effects on other AA-regulated genes, such as ASNS (3, 72), which are regulated by

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