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Elevated Mitochondrial Reactive Oxygen Species Generation Affects the Immune Response via Hypoxia-Inducible Factor-1α in Long-Lived Mclk1+/− Mouse Mutants

Dantong Wang,* Danielle Malo,† and Siegfried Hekimi*

Mitochondrial reactive oxygen species (ROS) are believed to stabilize hypoxia-inducible factor (HIF)-1α, a transcriptional regulator of the immune response. Mclk1 encodes a mitochondrial protein that is necessary for ubiquinone biosynthesis. Heterozygote Mclk1+/− mutant mice are long-lived despite increased mitochondrial ROS and decreased energy metabolism. In this study, Mclk1+/− mutant mice in the C57BL/6J background displayed increased basal and induced expression of HIF-1α in liver and macrophages in association with elevated expression of inflammatory cytokines, in particular TNF-α. Mutant macrophages showed increased classical and decreased alternative activation, and mutant mice were hypersensitive to LPS. Consistent with these observations in vivo, knock-down of Mclk1 in murine RAW264.7 macrophage-like cells induced increased mitochondrial ROS as well as elevated expression of HIF-1α and secretion of TNF-α. We used an antioxidant peptide targeted to mitochondria to show that altered ROS metabolism is necessary for the enhanced expression of HIF-1α, which, in turn, is necessary for increased TNF-α secretion. These findings provide in vivo evidence for the action of mitochondrial ROS on HIF-1α activity and demonstrate that changes in mitochondrial function within physiologically tolerable limits modulate the immune response. Our results further suggest that altered immune function through a limited increase in HIF-1α expression can positively impact animal longevity. The Journal of Immunology, 2010, 184: 582–590.
We sought to take advantage of \( Mckl1^{+/−} \) mice as an in vivo model of increased mitochondrial oxidative stress to further test the evidence that relates mitochondrial function to the control of HIF-\( \alpha \)-expression. Furthermore, we attempted to reconcile the positive features of the phenotype of \( Mckl1^{+/−} \) mutants with our observation of defective mitochondria in these animals. We focused on the immune system because of the known links between inflammation and oxidative stress and because immune function is crucial on the immune system because of the known links between inflammation and oxidative stress and because immune function is crucial.

### Materials and Methods

#### Animals and cells

The \( Mckl1 \) knockout mutants were described previously (18, 20) and were maintained in the heterozygous state. The C57BL/6 strain was derived from the SV129S6 strain by crossing \( \geq 10 \) times to C57BL/6 mice obtained from The Jackson Laboratory (Bar Harbor, ME). All procedures were approved by McGill University’s Animal Care and Ethics committee. RAW264.7 cells were purchased from the American Type Culture Collection and maintained in complete medium, containing DMEM (Invitrogen, Carlsbad, CA), in the presence of 10% FBS and penicillin (50 U/ml)-streptomycin (50 \( \mu \)g/ml).

#### Reagents

TNF-\( \alpha \) and IL-6 ELISA kits and Luminex multiple cytokine assay kits were purchased from R&D Systems (Minneapolis, MN) and BioSource International (Camarillo, CA), respectively. TRIZol reagent was purchased from Invitrogen; the RNeasy Kit, Omniscript RT Kit, and QuantiTect SYBR Green PCR Kit were purchased from Qiagen (Valencia, CA). Liquid alanine aminotransferase (ALT) Reagent Set was purchased from POINTE Scientific (Canton, MI). MitoSOX Red was from Invitrogen/Molecular Probes (Eugene, OR). LPS (L6529), fluorescent latex beads (L1278), and M-CSF (M9170) were purchased from Sigma-Aldrich (St. Louis, MO). The Cytosol Fractionation Kit and NF-\( \kappa \)B polyclonal Ab were purchased from Cell Signaling Technology (Danvers, MA). Cytosol Fractionation Kit and NF-\( \kappa \)B polyclonal Ab were purchased from Cell Signaling Technology (Danvers, MA). LPS was injected i.p. into 10–12-wk-old C57BL/6 male mice at doses of 0.01 or 1 mg/kg. Mice were anesthetized and sacrificed 2 h after injection. Blood samples obtained by cardiac puncture were preserved in EDTA-containing tubes and centrifuged at 8000 rpm at 4°C for 10 min. The supernatant was aliquoted at 50 \( \mu \)l/tube and stored at −80°C until use. Tissue samples were snap-frozen immediately in liquid nitrogen and kept at −80°C until use.

#### Western blotting and Real-time PCR

Peritoneal macrophages were cultured in complete medium for 24 h. After washing twice with PBS, the \( H_2O_2 \) concentration was measured using an Amplex Red Hydrogen Peroxide Assay Kit, according to the manufacturer’s instruction. All measurements were performed in duplicate. For the phagocytosis assay, peritoneal macrophages were incubated in the presence of latex beads with orange fluorescence for 30 min at a concentration of 1:1000. After fixation, the fluorescence was checked by FACS. For the bacteria-induced cytotoxicity assay, exponential growing phase \( Escherichia coli \) (DH10B) were cocultured with macrophages at two concentrations (yielding two multiplicities of infection [MOI]). A Cytotox ONE kit was used to quantify bacteria-induced cell death. The release of lactate dehydrogenase of bacteria-treated cells was normalized by untreated controls. All measurements were performed in duplicate, according to the manufacturer’s instructions. For the short peptide treatment, the freshly prepared peritoneal macrophages were cultured with 5 \( \mu \)g SS20 or SS31 for 24 h. The TNF-\( \alpha \) concentration of the medium was measured by ELISA.

#### RNAi knockdown experiments

RAW264.7 cells were treated at a concentration of 10\(^{5}\) cells/ml in serum-free medium using RNAiMAX reagent, according to the manufacturer’s instructions. Forty-eight hours after treatment, RNA was extracted using TRIzol reagent, and nuclear protein was extracted using Lysis buffer containing tubes and centrifuged at 8000 rpm at 4°C for 10 min. The supernatant was aliquoted at 50 \( \mu \)l/tube and stored at −80°C until use. Tissue samples were snap-frozen immediately in liquid nitrogen and kept at −80°C until use.
subjected to FACS analysis. For the LPS challenge experiment, cells were treated with siRNAs for 48 h and then incubated with 50 ng/ml LPS for an additional 90 min. For short-peptide treatment experiments, cells were treated with siRNAs against Mclk1 for 24 h before adding the short peptides to the medium to a final concentration 5 μM, and cultured for another 24 h before analysis. The sequences of all RNAs can be obtained upon request.

Statistical methods

Western band density was determined using Scion Image software (Scion). Data and graphs were processed using GraphPad Prism 5 software. Paired or unpaired t tests were used. The Welch correction was applied when the variances were different. Bar graphs are expressed as mean ± SE.

Results

Increased basal and LPS-induced HIF-1α expression in liver

We measured the level of HIF-1α in liver nuclei of Mclk1+/− mutants and control littermates and found that it was significantly elevated (Fig. 1A, 1B). The same samples were used to determine the level of RelA, a component of the transcription factor NF-κB, because of its close functional relationship with HIF-1α and its importance in innate immune function. The expression level of RelA was variable in both genotypes, but, on average, it was not significantly elevated in the mutants (Fig. 1A, 1B). However, there was a strong correlation between the levels of HIF-1α and the RelA expression in each sample from Mclk1+/− mutants but not in those from the Mclk1−/− controls, suggesting that RelA was elevated in at least some mutant animals (Fig. 1C).

RelA and HIF-1α can be induced by LPS (9, 10). To further study whether the altered expression of RelA and HIF-1α in Mclk1+/− mutants was likely to affect their immune function, we measured the level of HIF-1α and the RelA expression in liver nuclear extracts from untreated mice and mice treated with a low dose of LPS (0.01 mg/kg) (Fig. 1A, 1D). The expression of HIF-1α was increased in comparison with untreated animals in both genotypes (compare Fig. 1B and 1D), but it remained substantially higher in the mutants compared with the controls (Fig. 1D). Furthermore, the expression of RelA after treatment became significantly higher, on average, in the mutants (Fig. 1D).

To evaluate whether a specific cell type, such as Kupffer cells, is responsible for the elevation of HIF-1α, we carried out immuno-

Increased basal and hypoxia-induced HIF-1α and RelA expression in macrophages

Macrophages are one of the principal cell types that form the innate immune system and shape its responsiveness. We studied peritoneal macrophages, which were harvested and cultured overnight before analysis. Because of the limited amount of protein that could be extracted from the macrophages of a single animal, macrophages from mice of each genotype were pooled. Three groups of three mice were used. Under these conditions, the level of RelA could be readily detected and was found to be significantly increased in the mutants (Fig. 2A, 2B). However, HIF-1α was barely detectable (Fig. 2A), and no difference could be scored (Fig. 2B). Nonetheless, to obtain information on the expression of HIF-1α and to overcome the limit of detection by Western blotting, we scored Hif1α mRNA levels by RT-PCR and observed a significant increase in the transcription of Hif1α in the mutants (Fig. 2C).

The expression of HIF-1α and RelA is likely lower in culture than in vivo because of the hypoxia that cells experience when cultured in atmospheric oxygen (21%), although this condition is called normoxia by convention. In fact, the oxygen tension in healthy tissues is believed to be ∼2.5–9%. Therefore, we wondered whether the increases we observed in Mclk1−/− mutants could be due to their different sensitivity to oxygen. To mimic in vivo oxygen concentration, we cultured the macrophages in a hypoxia chamber with 5% oxygen overnight. The levels of both proteins in both genotypes increased in response to this relative hypoxia, and the levels of both proteins were higher in the mutants (Fig. 2A, 2D). In fact, the increased level of HIF-1α in Mclk1−/− mutants could now be scored. Thus, HIF-1α and RelA are expressed at higher levels in Mclk1−/− mutant livers treated with...
LPS and in mutant macrophages under relative hypoxia. The increased expression of these two transcription factors that control inflammation suggest that *Mclk1*+/− mutants might exhibit an enhanced inflammatory response.

**Increased classic activation of macrophages**

We tested whether mutant macrophages were activated, with the typical increased antibacterial and inflammatory effector functions, as a result of the increased expression of HIF-1α and NF-κB (10, 12, 24, 25). We used peritoneal macrophages cultured overnight to measure *Tnfa* mRNA expression by quantitative RT-PCR (Fig. 3A), as well as TNF-α and H2O2 secretion (Fig. 3B, 3C). All three measures were significantly elevated in the *Mclk1*+/− cells. We also tested bacteria-induced cell death, because increased resistance is expected from activated macrophages (26, 27). In fact, *Mclk1*+/− cells were more resistant at two different MOI (Fig. 3D). Lastly, we tested the phagocytic capacity of macrophages by scoring latex bead uptake (28), which was again increased in *Mclk1*+/− cells (Fig. 3E). This increase in classic macrophage activation is consistent with the increased level of HIF-1α expression.

**Resistance to inhibition of classic activation by IL-4**

IL-4 favors alternative macrophage activation and inhibits classic activation (15, 29). It was reported that the inhibitory effect of IL-4 on classic macrophage activation of BMDMs is dependent on intact mitochondrial function (17). Therefore, we wondered whether the *Mclk1*+/− mutant phenotype, which, in addition to increasing mitochondrial ROS, reduces mitochondrial oxidative phosphorylation (19), would affect the sensitivity of macrophages to IL-4. BMDMs from each genotype were prepared from the femur and tibia and grown for 3 d in the presence of 10 ng/ml M-CSF. After stimulation with LPS (2.5 ng/ml) and IFN-γ (1 U/ml), IL-4 treatment (5 ng/ml) of wild-type cells reduced the secretion of TNF-α and IL-6 to 55% and 41%, respectively, of that of untreated cells (i.e., 45% and 59% inhibition; Fig. 3F). In contrast, IL-4 treatment of *Mclk1*+/− mutant cells only reduced the secretion of TNF-α and IL-6 to 74% and 60%, respectively, of that of untreated cells (i.e., 26% and 40% inhibition; Fig. 3F). These findings suggest that the activated, inflammatory phenotype that we detected in *Mclk1*+/− mutant macrophages is the result of the effect of high mitochondrial ROS on HIF-1α expression and a reduced ability to differentiate along the alternative path of activation as the result of reduced oxidative phosphorylation.

It was reported that PPARγ coactivator 1β (PGC-1β) primes macrophages for alternative activation and strongly inhibits proinflammatory cytokine production (17). Therefore, we used quantitative RT-PCR to investigate whether the expression of PPARγ coactivator 1β was altered in the liver of LPS-treated mutant animals (0.01 mg/kg) and in mutant macrophages; no significant effect was observed (Supplemental Fig. 1B, 1C).

The increase in classic activation and the resistance to the effects of IL-4 in the macrophages of *Mclk1*+/− mice predicts a decrease in arginase activity and Arg1 gene expression, which is necessary for collagen synthesis during fibrosis, and is typical of alternative activation (15). As expected, we found a significant decrease in arginase activity (Fig. 3G) and Arg1 gene expression (Fig. 3H) in *Mclk1*+/− macrophages.

**Elevation of the plasma levels of inflammatory cytokines before and after treatment with a low dose of LPS**

The elevated levels of expression of HIF-1α and RelA in liver and peritoneal macrophages, as well as the partial resistance of macrophages to the inhibition of inflammatory cytokine secretion by IL-4 in vitro, predict an altered inflammatory response in *Mclk1*+/− mutants. We first used quantitative RT-PCR to measure the expression of the inflammatory cytokine genes *Tnfa* and Il6 in the liver of naive animals (Fig. 4A, 4B). The expression levels of both cytokines were increased in *Mclk1*+/− mutants, but only the increase observed for *Tnfa* reached statistical significance. Similarly, the plasma levels of both cytokines, measured by ELISA, seemed to be elevated (Fig. 4C, 4D), but the differences did not reach significance; this is likely due to the very low levels found in unstimulated animals and to the large animal-to-animal variability that we always observe in the mutants but not in the wild-type (see below).

Because the expression of HIF-1α was higher in *Mclk1*+/− mice after challenge with 0.01 mg/kg LPS, we treated animals in the same way before testing the circulating levels of four inflammatory cytokines (TNF-α, IL-1β, IL-6, and GM-CSF) and six.

**FIGURE 2.** Increased basal and hypoxia-induced HIF-1α and RelA expression in peritoneal macrophages of *Mclk1*+/− mice. A, A representative example of pools of three mice for each genotype were subjected to immunoblot analysis. B, Nuclear HIF-1α and RelA protein levels from three independent experiments under normoxia. C, *Hif1a* mRNA expression in peritoneal macrophages in 21% oxygen measured by quantitative RT-PCR (n = 8 for each genotype). D, Nuclear HIF-1α and RelA protein levels from four independent experiments under hypoxia. *p* < 0.05; paired *t* test.
Th1/Th2 cytokines (IFN-γ, IL-2, -4, -5, -10, and -12), using the Luminex multiple cytokine assay (BioSource). As shown in Fig. 4E and Supplemental Table I, significantly more TNF-α, IL-6 and -12, and GM-CSF were found in Mclk1+/− mice, on average. The elevations of plasma TNF-α and IL-6 in Mclk1+/− mice were confirmed by ELISA (Supplemental Fig. 2). The level of IL-1β was also elevated (Fig. 4E, Supplemental Table I), but the difference did not reach statistical significance.

We observed a great spread of values for cytokine levels in the mutants, with many animals having cytokine levels that were not different from those of wild-type animals. However, the levels of cytokines in the mutants were correlated with each other. Fig. 4F and 4G illustrate the correlations between the levels of TNF-α and IL-6 and TNF-α and IL-12, respectively. Animals that have elevated levels of TNF-α also have elevated levels of IL-6 and -12. This indicates that some mutant animals had higher levels of several cytokines, but other animals did not have elevated cytokines. In conclusion, the inflammatory response in Mclk1+/− mice is variable but is hypersensitive to low levels of LPS in many individual animals, and cytokine levels also seem to be spontaneously elevated in some animals.

No injury and enhanced resistance to infection from the hypersensitive inflammatory response

The inflammatory response can result in chronic and acute tissue damage. Plasma ALT activity, a measure of tissue damage, was used to estimate the extent of such damage in naive animals as well as in animals 2 or 24 h after treatment with a high dose of LPS (1 mg/kg) (Fig. 5A). No significant differences in ALT levels between the genotypes were detected. We also evaluated the effect of 1 mg/kg LPS on cytokine levels 2 h after treatment. As expected, the levels of several cytokines were significantly higher than after treatment with 0.01 mg/kg LPS, but there was no significant difference between the genotypes with this harsher treatment (Supplemental Table I). The inflammatory reaction triggered by treatment with a high level of LPS represents an important stress, and animals treated in this way can lose a substantial fraction of their body weight (30). We determined the effect of 1 mg/kg of LPS on body weight 24 h after the treatment (Fig. 5B) and observed that Mclk1+/− mutants lost a significantly smaller fraction of their body weight as a consequence of their response. Thus, the hair-trigger inflammatory response that we observed in Mclk1+/− mutants is not obviously deleterious at low or high levels of LPS stimulation.

**Reduction of Mclk1 expression in RAW264.7 cells increases nuclear HIF-1α and Hif1α-dependent TNF-α secretion**

To verify the causal links suggested by the analysis of the phenotype of Mclk1+/− mice, we used the RAW264.7 mouse macrophage-like cell line, which is a model for the study of macrophages, HIF-1α, and mitochondria (10, 31–35). The levels of nuclear HIF-1α were significantly increased upon siRNA knockdown of Mclk1 (Figs. 6A, 6B, 7B). Mclk1 knockdown also significantly elevated TNF-α levels in the medium of the cells after 48 h without LPS treatment or 1.5 h after stimulation with 50 ng/ml LPS (Figs. 6C, 6D, 7C). These increases in TNF-α were entirely abolished by concomitant knockdown of Hif1α (Fig. 6C, 6D). The levels of Hif1α mRNA were also monitored in these experiments on TNF-α secretion (Fig. 6E, 6F). The effect of Mclk1 knockdown on HIF-1α seems to be post-transcriptional, because Hif1α mRNA levels were not altered by Mclk1 knockdown, but the effects on TNF-α were clearly Hif1α expression dependent.
The effect of reduced Mclkl expression in RAW264.7 cells on HIF-1α expression and TNF-α secretion depends on changes in mitochondrial ROS metabolism

We first established that Mclkl knockdown in RAW264.7 cells increased mitochondrial ROS levels as expected from the phenotype of Mclkl<sup>+++/−</sup> mice. We used MitoSOX Red fluorescence to monitor mitochondrial superoxide levels. Superoxide is the ROS produced initially as a result of mitochondrial electron transport, and its production is increased as the result of inefficient or abnormal function of the mitochondrial respiratory chain. Mitochondrial superoxide is detoxified by superoxide dismutase (SOD) 2, the mitochondrial Cu/Zn SOD. Therefore, we used siRNA knockdown of Sod2 as a control, because this would be expected to increase mitochondrial superoxide levels (Fig. 7A). Mclkl and Sod2 knockdown led to a clear increase in MitoSOX Red fluorescence. Although superoxide is the ROS initially produced in the mitochondria, it is rapidly detoxified by SOD2 into peroxide (H₂O₂), which, in contrast to superoxide, is able to cross membranes. Most antioxidants are not specific for the mitochondria or for a single ROS species. Thus, they exercise powerful effects on many cellular functions that can be affected by ROS or the redox state and, therefore, could affect HIF-1α and TNF-α levels in a variety of indirect ways. Thus, to test whether the altered ROS metabolism induced by Mclkl knockdown is responsible for the increase in HIF-1α and TNF-α levels, we used a mitochondrially targeted H₂O₂-specific antioxidant peptide (Szteo-Schiller peptide SS31) and its inactive homolog (SS20) as a control (36, 37). SS31 treatment, but not SS20 treatment, fully abolished the effect of Mclkl on HIF-1α and TNF-α (Fig. 7B, 7C). Comparable effects were also obtained in peritoneal macrophages (Fig. 7D). These findings fully support the notion that a ROS-based signal from mitochondria, likely H₂O₂ itself, can regulate HIF-1α levels and that the effects of reduced MCLKL1 levels in mice and cultured cells is mediated by altered mitochondrial ROS metabolism.

Discussion

Several studies documented that ROS originating from the mitochondria in response to hypoxia, likely H₂O₂, are involved in the stabilization of HIF-1α (2–4). We showed in this study that Mclkl<sup>+++/−</sup> mouse mutants, which sustained elevated mitochondrial oxidative stress (19), exhibited elevated levels of HIF-1α: 1) HIF-1α is elevated in the livers of mutant mice, even in the absence of any inducing treatment; 2) treatment of the animals with a very low dose of LPS triggered an increase in HIF-1α in wild-type and mutant mice but to a much greater extent in the mutants; and 3) the effect of mild hypoxia on HIF-1α was much more pronounced in peritoneal macrophages from Mclkl<sup>+++/−</sup> mice than from sibling controls. Furthermore, RAW264.7 macrophage-like cells in which Mclkl had been knocked down by siRNA treatment displayed elevated HIF-1α levels that required elevated mitochondrial H₂O₂. To our knowledge, these findings provide the first evidence for the action of mitochondrial ROS on HIF-1α expression and function in an intact animal and provide a model to study these mechanisms further. Although we investigated the consequences of a reduction in MCLKL1 levels on HIF-1α, the
level of mitochondrial changes observed in \( Mclk1^{+/2} \) could readily be produced by other conditions, such as toxins, anticancer drugs, hypoxia, hyperthermia, or deliberate pharmacological intervention. A number of observations with \( Mclk1^{+/2} \) mutants suggest a mechanism for the amplification of the effect on HIF-1 of a potentially small initial increase in mitochondrial ROS generation. These observations include increased classic macrophage activation; partial resistance to alternative macrophage activation; increased expression of inflammatory cytokines; increased expression of the NF-κB subunit RelA, including in macrophages; and increased \( Hif1a \) gene expression in macrophages. The mechanism of amplification might include the following steps: HIF-1a activates macrophages, and more generally favors the inflammatory response (11), which leads to the secretion of cytokines, such as TNF-α, IL-1β, and others, which, in turn, activates the expression of NF-κB (38), which is a transcriptional activator of \( Hif1a \) (13). We did not observe a transcriptional effect in RAW264.7 cells, which is consistent with the notion that the effect of \( Hif1a \) transcription is a secondary one via cytokine expression in the animals. The reduction of mitochondrial oxidative phosphorylation that is observed in \( Mclk1^{+/2} \) mutants (19) might further contribute to the amplification of the effect of mitochondrial ROS on HIF-1 by partially inhibiting the effect of IL-4 in triggering alternative over classic activation of macrophages, because the net effect of this inhibition should be an increase in classic activation. To fully establish a causal relationship between the increased levels of HIF-1a and the enhanced inflammatory response that we observed in \( Mclk1^{+/2} \) mutants, we plan to study the genetic interactions between \( Mclk1 \) and \( Hif1a \) in double-mutant combinations.

The inflammatory response is a powerful weapon against infection. As such, it can also have deleterious effects, such as the temporary incapacitation of the organism, as well as inducing tissue damage. \( Mclk1^{+/2} \) mutants show a tendency for elevated markers of inflammation in the absence of experimental stimulation. Possibly, this is due to a stronger than normal reaction to the numerous minor infections that mammals sustain and combat successfully on their own.
The aging process impairs immune system function (39). Thus, under imperfectly aseptic conditions, part of the mortality of caged mice is likely due to infections by microorganisms to which they have good resistance when young, but which decreases when weakened by senescence. Therefore, the increased lifespan of Mclk1+/−/− could be the result of greater resistance to infectious microorganisms. Interestingly, in the survival experiment with Mclk1+/−/− mutants conducted previously in the background used in this study (C57BL/6J), we observed a surprisingly short lifespan for the control Mclk1+/+/+ mice (20) compared with previously reported results for animals in this background (40, 41). This suggests that environmental conditions, such as exposure to microorganisms, were limiting the survival of the wild-type animals in this particular experiment, yet Mclk1+/−/− mutants had significantly better survival than their Mclk1+/+/+ siblings, possibly because of their hair-trigger immune response.

We hypothesize that another mechanism linked to infection and immunity, beyond actual better immediate survival from infection, could contribute to the greater lifespan of the mutants. The inflammatory reaction, and the subsequent development of the immune response, protects against infection but also injures the tissues of the infected animals. Thus, the immune response needs to be terminated and the tissue repaired. However, these processes are imperfect; therefore, infection can lead to chronic inflammation and fibrosis (42). Animals that sustain repeated serious infections and the subsequent immune response might suffer from a gradual accumulation of fibrotic lesions, as well as suffer more severely from age-dependent diseases, many of which have the characteristic of chronic inflammation. The link between inflammation and the aged phenotype has been studied most extensively in humans (43). By producing a relatively strong reaction at the slightest sign of infection, the hair-trigger inflammatory response of Mclk1+/−/− mutants might prevent severe or chronic infection. Such a hair-trigger response might not be favored by natural selection because of various energetic and behavioral costs to the animals, but it might allow Mclk1+/−/− mutants, which are fed and sheltered from predators in the animal facility, to sustain fewer severe infections and the subsequent damage inflicted by the microorganisms and the full run of the immune reaction against them. Thus, in the long-term, a hair-trigger response might lead to a slower accumulation of permanent damage or chronic inflammation due to infection. These considerations suggest that the normal immune response contributes to physiological aging, as assessed by biomarkers of oxidative stress, which are also increased by inflammatory states. Therefore, it is of interest that Mclk1+/−/− mutants display dramatically improved global biomarkers of aging (21). This suggests the possibility that the accumulation of oxidative damage that is observed with aging is, in part, the consequence of the damage resulting from chronic infection and inflammation (44).

In humans, an association has been observed between lower childhood mortality and an increase in longevity that is typical of cohorts born closer to modern times (45). The low childhood mortality has been interpreted as resulting from a lower rate of infection, possibly due to better sanitation. This, in turn, has been interpreted as pointing to the deleterious consequences of inflammation for longevity, especially in light of the fact that many age-dependent diseases have an inflammatory component. Our findings suggest the possibility of a different interpretation of the same observations. The low rate of childhood infection and mortality might be the result of a stronger or more sensitive immune response in addition to better sanitation. Indeed, the characteristics of the immune response also depend on environmental factors, such as nutrition (46). Thus, a hair-trigger inflammatory response that results in a low rate of severe infection throughout life might ultimately favor longevity for humans as well. It is possible that the inflammatory age-dependent diseases that are the

**FIGURE 7.** The effect of reduced Mclk1 expression on HIF-1α expression and TNF-α secretion depends on changes in mitochondrial ROS. A, Mitochondrial superoxide levels measured by MitoSOX Red after siRNA treatment. siRNA against Sod2 was used as a positive control. B, Nuclear HIF-1α levels after Mclk1 knockdown and controls, and treatment with an antioxidant peptide (SS31), and a control (SS20), from 4 independent experiments. The signal ratios between HIF-1α and TBP are plotted. Paired t tests were used. C, TNF-α concentrations in the medium of RAW cells under the same conditions as in (B) (n = 5). D, TNF-α concentrations in the medium of peritoneal macrophages from wild-type and Mclk1+/−/− mice treated with SS20 and SS31, as in B. *p < 0.05; t test.
most prevalent diseases in long-lived modern humans are a trade-off that limits further increases in lifespan.

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Disclosures

The authors have no financial conflicts to report.

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