Research Article

Apo A1 Mimetic Rescues the Diabetic Phenotype of HO-2 Knockout Mice via an Increase in HO-1 Adiponectin and LKBI Signaling Pathway

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Insulin resistance, with adipose tissue dysfunction, is one of the hallmarks of metabolic syndrome. We have reported a metabolic syndrome-like phenotype in heme oxygenase (HO)-2 knockout mice, which presented with concurrent HO-1 deficiency and were amenable to rescue by an EET analog. Apo A-I mimetic peptides, such as L-4F, have been shown to induce HO-1 expression and decrease oxidative stress and adiposity. In this study we aimed to characterize alleviatory effects of HO-1 induction (if any) on metabolic imbalance observed in HO-2 KO mice. In this regard, HO-2(−/−) mice were injected with 2 mg/kg/day L-4F, or vehicle, i.p., for 6 weeks. As before, compared to WT animals, the HO-2 null mice were obese, displayed insulin resistance, and had elevated blood pressure. These changes were accompanied by enhanced tissue (hepatic) oxidative stress along with attenuation of HO-1 expression and activity and reduced adiponectin, pAMPK, and LKB1 expression. Treatment with L-4F restored HO-1 expression and activity and increased adiponectin, LKB1, and pAMPK in the HO-2(−/−) mice. These alterations resulted in a decrease in blood pressure, insulin resistance, blood glucose, and adiposity. Taken together, our results show that a deficient HO-1 response, in a state with reduced HO-2 basal levels, is accompanied by disruption of metabolic homeostasis which is successfully restored by an HO-1 inducer.

1. Introduction

Obesity, metabolic syndrome, and associated insulin resistance are major contributors to cardiovascular disease, the leading cause of mortality in the United States [1]. Insulin resistance is characterized by hyperglycemia and increased lipolysis and free fatty acid levels and increased hepatic triglyceride secretion and sterol-regulatory element-binding protein-1 (SREBP-1) [2, 3]. SREBPs are transcription factors known to regulate genes involved in fatty acid and cholesterol synthesis and are regulated by pAMPK [3, 4]. AMPK is phosphorylated and activated by the major kinase, LKB1, and acts as a metabolic checkpoint that is suppressed in hyperglycemic conditions [4–8]. Hyperglycemia and associated increase in reactive oxygen species (ROS) are known to decrease HO levels [1, 9, 10]. There are two forms of HO, the inducible HO-1 and the constitutively expressed HO-2 [4, 11]. HO-1 and -2 catabolize heme into equimolar concentrations of carbon monoxide, bilirubin, and free iron, generating an antioxidant effect and increasing nitric oxide (NO) bioavailability and providing cardiovascular protection [4, 11]. HO-1 is the major cytoprotective moiety of the HO system because of its rapid inducibility by a broad spectrum of compounds and conditions including stress. However, recent studies using HO-2(−/−) mice suggest that HO-2 is also critical for cellular homeostasis and for upregulation of HO-1 [4, 11]. When HO-1 increases, levels of antioxidant and anti-inflammatory molecules increase and the level of reactive oxygen species (ROS) decreases [9]. The benefits of increased levels of HO-1 protein include the prevention of
high blood pressure, decreased vasoconstrictors, increased vasodi-
ators, and the inhibition of oxidative stress [1, 10, 12].

The effects of HO are also associated with an increase in
adiponectin, a protein hormone that modulates many meta-
bolic processes and can improve cardiovascular function
while downregulating proinflammatory factors [1, 9, 13, 14].
Adiponectin exists in three different forms trimer, hexamer,
and high molecular weight (HMW) adiponectin
being the form that attenuates cardio-vascular disease [1, 14].
In both, obese subjects and animals, the plasma levels of
adiponectin are inversely related to insulin sensitivity [4, 13,
15, 16]. The upregulation of HO-1 is associated with an
increase in adiponectin levels and correlates with decreased
inflammatory cytokines, IL-1, IL-6, and TNFα [1, 9].

Recently developed HO-2 null mice have displayed char-
acteristics of a metabolic syndrome-like phenotype with en-
hanced systemic inflammatory and oxidative stress response.
Curiously, these mice also demonstrate a failure to induce
stress-dependent HO-1 upregulation along with suppression
of adiponectin levels. That attenuated HO-1 upregulation in
an HO-2 null mouse is accompanied by metabolic imbalance
led us to examine the effects of an HO-1 inducer in such a
setting. The apo-A1 mimetic peptide, L-4f, was administered
to HO-2 null mice so as to rescue HO-1 expression.
This apo-A1-mimetic peptide was synthesized from amino
acids that improved the ability of HDL to protect LDL
against oxidation in animals with atherosclerosis [2]. L-4F
treatment resulted in reduced adiposity, evident by decreased
visceral fat content, in conjunction with improved energy
balance and metabolic homeostasis in HO-2 null mice. These
changes were further characterized by increases in HO-1 and
adiponectin levels along with enhanced cellular expression of
LKB1-pAMPK in the liver tissues.

2. Materials and Methods

2.1. Animal Protocol. All animal experiments followed an
institutionally approved protocol in accordance with the NIH
Guide for the Care and Use of Laboratory Animals. The HO-2
null mice are direct descendents of the HO-2 mutants
produced [17]. These well-characterized HO-2 null mice are direct descendents of the HO-2 mutants
2 null mice are direct descendents of the HO-2 mutants
Guide for the Care and Use of Laboratory Animals. The HO-
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2.2. Western Blot Analysis. Frozen hepatic samples were pul-
erized in T-PER (ThermoFisher Scientific, Rockford, IL,
USA) homogenization buffer, rotated for 1 hour at 4°C, and
then centrifuged at 12,000 rpm for 25 minutes at 4°C. The
supernatant was collected and protein was quantified using
the BCA protein assay (Pierce Biotechnology, Inc., Woburn,
MA). Protein expression analysis was performed through
immunoblotting with antibodies against HO-1 (Stressgen
Biotechnologies Corp., Victoria, BC, Canada), adiponectin,
pAKT, pAKT, pAMPK, and AMPK (Cell Signaling Technology,
Inc. Beverly, MA, USA) were used. Imaging and quantifica-
tion were done using the Odyssey imaging system (Li-Cor
Biosciences, Lincoln, NE, USA).

2.3. Real-Time Quantitative PCR. Total RNA was recovered
from liver following the Perfect Pure Tissue Kit (5Prime,
IN Gaithersburg, MD, USA) RNA extraction protocol
with DNase treatment. cDNA was made using the Improm
Reverse Transcriptase kit (Promega, Madison, WI, USA). Prim-
er sequences for mouse HO-1 were 5’-CAAGCCCCA-
CACATTCAAC-3’ and 5’-TCAGGTGTCACTCTCCAGA-
GTGTC-3’, adiponectin 5’-AGCGGCCATATGATTCTGCT-
CAT-3’ and 5’-TGCGGTCAATATGATTCTGTC-3’, AMPK 5’-
CGCACAGGCCAAAG-3’ and 5’-AGAGACTTGGGCTTCGAG-
AGACCTTA-3’ and 5’-CTCGCCAGCTTTTCTCC-3’, AKT 5’-
GAACCTGTCTCTGAGAATCTGAG-3’ and 5’-
GTGGGCTTGAGAATCTGTCG-3’, and GAPDH 5’-
CCAGGTGTGTCCTCGGCGTC-3’ and 5’-ATACGAGGAA-
ATTGAGCTTGCAAAAT-3’. The thermal cycling condi-
tions were 95°C for 20 seconds followed by 40 cycles of 95°C
for 3 minutes, 60°C for 30 seconds, and finally 95°C for 15
seconds, 60°C for 1 minute, and 95°C for 15 seconds.

2.4. O2⁻ Production. Liver samples were placed in scintil-
lation vials (2 per vial) containing 1 mL of Krebs-HEPES
buffer, pH 7.4, and lucigenin (5 μmol/L) for 30 min at 37°C.
Lucigenin chemiluminescence was measured in a liquid scin-
tillation counter (LS6000TA, Beckman Instruments) and su-
peroxide production quantified as previously described [2].

2.5. Glucose and Insulin Tolerance Tests. After 6 h fast, mice
were injected intraperitoneally with glucose (2.0 g/kg body
weight). Blood samples were taken at various time points (0–
120 min), and blood glucose levels and serum insulin levels
were measured. For determination of insulin tolerance, mice
were injected intraperitoneally with insulin (2.0 U/kg). Blood
samples were taken at various time points (0–90 min), and blood glucose levels were measured.

2.6. Measurement of HO Activity. Tissue HO activity, in liver samples from WT, HO-2(−/−) treated and untreated mice, was assayed as described previously [18, 19] using a technique in which bilirubin, the end product of heme degradation, was extracted with chloroform, and its concentration was determined spectrophotometrically (dual UV/VIS beam spectrophotometer lambda 25; PerkinElmer Life and Analytical Sciences, Wellesley, MA, USA) using the difference in absorbance at a wavelength from λ460 to λ530 nm with an absorption coefficient of 40 mM$^{-1}$ cm$^{-1}$. Under these conditions, HO activity was linear with protein concentration, time-dependent, and substrate-dependent [18, 19].

2.7. Statistical Analyses. Statistical significance between experimental groups was determined by the Fisher method of analysis of multiple comparisons ($P<0.05$). For comparison between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired $t$-test for two groups.

3. Results

3.1. Effect of HO-2 Deletion on Body Weight, Fat Content, Blood Pressure, and Metabolic Parameters. In Figure 1(a) we show that HO-2 deletion significantly increased body weight when compared to WT mice and was reversed after six weeks of L4-F treatment. A similar pattern was observed in weight reduction of subcutaneous and visceral fat by administration of L4-F in HO-2(−/−) mice as shown in Figures 1(b) and 1(c).

Furthermore, we determined that the random blood glucose levels in the HO-2 null mice were significantly increased compared to WT and were reversed back to normal baseline with L4-F treatment (Figure 2(a)). Both systolic and diastolic blood pressures were significantly elevated in HO-2 null mice as compared to WTs ($P<0.05$) (Figure 2(b)). This increased body weight, adiposity, and elevated blood pressure suggests metabolic syndrome like phenotype in HO-2 KO mice, which was successfully reversed by HO-1 induction.

To investigate if the metabolic syndrome, observed in HO-2 null mice, is associated with insulin resistance, we performed insulin sensitivity and glucose tolerance tests. Insulin administration to WT, HO-2 null, and L4-F treated HO-2 null mice produced a rapid decrease in glucose levels in the WT and L-4F treated HO-2 KO mice compared to HO-2 null mice.
null ($P < 0.05$), suggesting improved sensitivity of HO-2 null mice with L-4F treatment, decreasing from 333.7 ± 6.0 mg/dL for HO-2 null mice to 84.7 ± 4.9 mg/dL in the L-4F treated HO-2 null mice (Figure 2(c)). Plasma glucose levels at all times were significantly elevated in the HO-2 null mice compared to the L-4F treated HO-2 null mice. Glucose administration to all mice rapidly increased the glucose level after 30 min and remained elevated in the HO-2 null mice, compared to the L4-F treated HO-2 null and WT mice which returned to initial levels at 120 min (Figure 2(d)).

### 3.2. Effect of HO-2 KO on Tissue Redox, HO-1 Expression, and Activity

Analysis of lucigenin-detectable chemiluminescence demonstrated enhanced oxidative stress in hepatic samples from HO-2 KO versus WT mice ($P < 0.05$). This increase in $\mathbb{O}_2^-$ generation was attenuated ($P < 0.05$) in HO-2 null mice treated with L-4f for 6 wks (Figure 3(a)). In Figure 3(b), we show that the HO-2 null mice express significantly ($P < 0.05$) lower levels of HO-1 as compared to WT mice; however levels are restored with L-4F treatment. A daily injection of L-4F for 6 weeks also resulted in a
significant increase in HO-1 mRNA levels compared to the HO-2 null mice (Figure 3(c)). HO-2 deletion impairs HO-1 inducibility leading to a decrease in HO activity. In Figure 3(d) we show that treatment with L-4F in the HO-2 null mice significantly increases HO-1 activity.

3.3. Effect of HO-2 KO on Hepatic Adiponectin and pLKB1/pAMPK Signaling. Western blot analysis demonstrated that HO-2 deletion is associated with significant decrease in the expression of adiponectin when compared to age-matched WT (Figure 4(a)). Treatment with L-4F increased these levels by 2-fold to levels significantly higher than those measured in HO-2 null mice (Figure 4(a)). Consistent with the changes in protein shown in Figure 4(a), we show with real-time PCR that adiponectin levels significantly increase with treatment with L-4F compared to untreated in the HO-2 null mice (Figure 4(b)). To elucidate the mechanism involved in the changes observed with L-4F treatment, we determined expression and activity of signaling pathways that may be involved in the process. Interestingly, the expression of activated AMPK was regulated by HO-2 deletion since the HO-2 null mice expressed significantly lower levels of pAMPK albeit normal levels of AMPK. L-4F restored the levels of pAMPK in the HO-2 null mice without affecting total AMPK levels (Figure 4(c)). Furthermore, there was a significant increase in LKB1 expression in the L-4F treated HO-2 null mice (Figure 4(d)), which suggests that the AMPK/LKB1 pathway could play a role in the L-4F mediated resource of HO-2(-/-) phenotype. In addition, to elucidate modulation of AKT-dependent pathways by HO-1 induction via L-4F, immunoblot assessment of pAKT/AKT was performed which exhibited enhanced (P < 0.05) pAKT/AKT levels in HO-2 KO (1.39 ± 0.12) versus WT (1.02 ± 0.09) mice. This effect of HO-2 deletion on pAKT expression was unaffected by L-4F administration in HO-2 null mice (1.46 ± 0.14).
4. Discussion

The data presented here shows that treatment of HO-2 null mice with L-4F rescues the key markers of metabolic syndrome via an increase in HO-1 and adiponectin through a signaling mechanism involving the LKB1/AMPK signaling pathway (Figure 5). Interestingly, L-4F had no effect on activated AKT (pAKT), suggesting selectivity of L-4F to pAMPK.

First key finding presented here is in line with earlier reports [4, 11] suggesting a role of HO-2 in mediating HO-1 upregulation. HO-2 null mice were characterized by disruption of metabolic homeostasis and displayed increased body weight, adiposity, insulin resistance with elevated blood pressure, and oxidative stress. Pathophysiological conditions such as these have historically been shown to be associated with increase in cellular defense mechanisms including HO-1 [20]. HO-2, a constitutively expressed isoform, supports sustenance of basal redox status in the cells, and its knockdown is not surprisingly met with oxidative stress. HO-1 induction, however, in this HO-2 knockdown state fails to occur even in the presence of added pathophysiological insult such as metabolic syndrome. These observations delineate the essential role of HO-2 in stress-induced HO-1 induction. This failure of HO-1 upregulation could further dampen
cellular defenses and contribute towards the phenotypic alteration observed in these animals. Restoration of HO-1 expression and activity accompanied by phenotype reversal further supports the role of deficient HO-1 in mediating, at least partly, clinicopathological alterations observed in HO-2 null state. Previous reports have documented physical interactions of the two HO isoforms [21], which could contribute towards HO-2-dependent induction of HO-1.

Second key observation of this study is the modulatory effect of heme-HO system on adiponectin and associated metabolic signaling pathways and their role in alleviating metabolic pathologies observed in an HO-2 KO state. One major marker of obesity is inflammation, which produces an excess of reactive oxygen species, specifically superoxide [11]. When there is chronic exposure to an excess of superoxide, adiponectin and HO-1 levels decrease significantly and contribute to the pathogenesis of insulin resistance [2, 4, 9, 12]. An increase in HO-1 levels increases adiponectin levels, which is known to possess a vascular protective role, preserve endothelial function, and improve insulin sensitivity through glucose uptake [1, 9]. Treatment with L-4F is shown to increase both HO-1 and adiponectin levels in vitro and in vivo [2, 15] while decreasing superoxide (Figure 3(a)), further supporting the idea that L-4F improves the phenotype in the metabolic syndrome mouse model through an increase in insulin sensitivity and glucose tolerance.

L-4F treatment significantly increased pAMPK and LKB1 levels, all associated with improved insulin sensitivity [15]. P-AMPK is known to act in the regulation of cell survival, protect against oxidative stress [15, 22–24], and, when activated, contribute to glucose transport, fatty acid oxidation, and increased mitochondrial function [2, 25]. It is known that crosstalk between AMPK and AKT can regulate nitric oxide bioavailability and vascular function [22, 23, 26]. However L-4F did not affect the protein expression or activation of AKT, suggesting a pathway more specific to AMPK. LKB1 is a serine-threonine kinase that directly phosphorylates AMPK and decreases lipogenesis [4, 5, 8]. We show that L-4F induces LKB1 in HO-2 null mice, indicating that HO-1 mediates the transcriptional regulation of LKB1 by L-4F to activate AMPK.

In conclusion, as depicted in the schematic (Figure 5), the upregulation of HO-1 and adiponectin levels by L-4F coincides with increased pAMPK and LKB1 levels, providing a signaling mechanism by which L-4F rescues the metabolic syndrome phenotype and improves energy balance. Thus, L-4F could provide as a beneficial drug treatment to complement conventional therapeutic of disease associated with disruption of metabolic homeostasis.

Abbreviations:

HO-1/HO-2: Heme oxygenase 1, 2
ROS: Reactive oxygen species
EC-SOD: Extracellular superoxide dismutase
NO: Nitric oxide
AKT: Protein kinase B
pAKT: Phosphorylated protein kinase B
AMPK: AMP-activated protein kinase
pAMPK: Phosphorylated AMP-activated protein kinase
O$_2^-$: Superoxide
LKB1: Serine/threonine kinase 11.
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