Received: 1 April 2023 | Revised: 19 July 2023 | Accepted: 21 July 2023

DOI: 10.1111/apha.14023

ACTA PHYSIOLOGICA

RESEARCH PAPER

Short-term dietary intervention improves endothelial dysfunction induced by high-fat feeding in mice through upregulation of the AMPK-CREB signaling pathway

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Funding information
Fundación Universitaria San Pablo CEU – Santander; Ministerio de Economía y Competitividad, Grant/Award Number: BFU2017-82565-C2-2-R; Grupos Universidad Complutense de Madrid, Grant/Award Number: GR-921641

Abstract

Aim: In addition to functioning as an energy sensor switch, AMPK plays a key role in the maintenance of cardiovascular homeostasis. However, obesity disrupts AMPK signaling, contributing to endothelial dysfunction and cardiovascular disease. This study aimed to elucidate if a short-term dietary intervention consisting in replacing the high-fat diet with a standard diet for 2 weeks could reverse obesity-induced endothelial dysfunction via AMPK-CREB activation.

Methods: For this, 5-week-old male C57BL6J mice were fed a standard (Chow) or a high-fat (HF) diet for 8 weeks. The HF diet was replaced by the chow diet for the last 2 weeks in half of HF mice, generating 3 groups: Chow, HF and HF-Chow. Vascular reactivity and western-blot assays were performed in the thoracic aorta.

Results: Returning to a chow diet significantly reduced body weight and glucose intolerance. Relaxant responses to acetylcholine and the AMPK activator (AICAR) were significantly impaired in HF mice but improved in HF-Chow mice. The protein levels of AMPKα, p-CREB and antioxidant systems (heme oxygenase-1 (HO-1) and catalase) were significantly reduced in HF but normalized in HF-Chow mice.

Conclusion: Improving dietary intake by replacing a HF diet with a standard diet improves AMPK-mediated responses due to the upregulation of the AMPK/CREB/HO-1 signaling pathway.

See related editorial: KD Nüsken, E Nüsken, 2023. Molecular effects of dietary interventions—A clinical perspective. *Acta Physiol*. (Oxf). e14050.

KEYWORDS
AMPK, CREB, dietary intervention, endothelial dysfunction, heme oxygenase-1

1 | INTRODUCTION

Obesity is considered as one of the biggest health challenges of the 21st century due to its associated comorbidities and mortality, and the elevated healthcare costs. The chronic intake of foods enriched in saturated fat and carbohydrates, the so called "*high-fat or western diets*", results in a sustained positive energetic balance and, consequently, in the development of obesity, one of the major risk factors for the development of cardiovascular disease (CVD).[1,2]

Several studies have pointed out caloric restriction (CR) and intermittent fasting (IF) as useful strategies to prevent the apparition of endothelial dysfunction, an initial step in the pathogenesis of obesity-related CVD (for review, see Savencu et al[3]). However, we have recently reported that even the intake of normocaloric diets based on easy-metabolizable carbohydrates, saturated fats, and reduced amounts of soluble fiber, monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) promotes endothelial function impairment in the murine thoracic aorta, which could be totally prevented with a healthy diet.[4] Therefore, the promotion of healthy dietary patterns, including an increased intake of foods enriched in fiber-rich complex carbohydrates, MUFA, and PUFA, is currently the most recommended strategy to prevent obesity-related CVD.[5]

Adenosine monophosphate-activated protein kinase (AMPK) acts as a critical metabolic regulator that is activated in response to cell stress (i.e., changes in the AMP-to-ATP ratio). Previous studies have shown that the phosphorylation of endothelial AMPKα$^{(Thr172)}$ triggers endothelial nitric oxide synthase (eNOS) activation, increasing NO release that induces vascular relaxation. AMPK also has a significant beneficial effect on oxidative stress. Indeed, it reduces NADPH-oxidase activity and increases the expression of antioxidant enzymes, such as catalase and manganese superoxide dismutase (SOD), contributing to preserving endothelial function.[6] Moreover, we recently demonstrated an important dysregulation of endothelial AMPK activity in obese rats[7] and that CR can improve endothelium-dependent relaxation in the thoracic aorta from Zucker *fa/fa* rats through the upregulation of the AMPK-eNOS-NO pathway.[6,8]

Cyclic AMP-response element binding protein (CREB) is a phosphorylation target of AMPK. Its activation (pCREB$^{(Ser\ 133)}$) exerts an important vascular protective role associated to increased expression of both antioxidant and anti-inflammatory genes, that is, heme oxygenase-1 (HO-1), that is upregulated in response to oxidative stress.[9,10] Nonetheless, the precise role of CREB in vascular regulation is not fully understood. Interestingly, HO-1 plays an important cytoprotective role in endothelial cells during periods of metabolic stress,[11] and its induction was shown to improve endothelial function in obese rats through the activation of the AMPK-PI3K/Akt-eNOS signaling pathway.[12] Moreover, reduced expression of aortic CREB has been found in mouse models of hypertension, atherosclerosis, and insulin resistance.[13] In this regard, a recent study has shown that celecoxib drives HO-1 expression in human endothelial cells through activation of the AMPK-CREB-Nrf2 pathway.[14]

Based on these findings, we hypothesize that replacement of HF feeding with a healthy dietary intake could induce the upregulation of the AMPK-CREB signaling pathway, thus increasing the expression of antioxidant systems, including the HO-1, and resulting in an improvement of endothelial function. Therefore, the aim of this study was to analyze the effect of a short dietary intervention of 2 weeks on: (i) body weight (BW) and adiposity, (ii) biochemical parameters, (iii) endothelial function and NO bioavailability, (iv) AMPK-mediated vascular responses, and (v) the protein levels and phosphorylation of AMPK and CREB.

2 | RESULTS

2.1 | The dietary intervention reversed the BW increase and glucose intolerance induced by HF feeding

After 8 weeks, HF mice exhibited a significant increase in BW (Figure 1B), caloric intake (Figure 1C) and the amount of white fat pads (including, PR-AT, Mes-AT, SC-AT, and PG-AT) and brown fat pads (PAT-AT and BAT) as shown in Table 1. Plasma cholesterol concentrations, determined in non-fasted conditions, were also significantly higher in HF mice (Table 1), but no changes were detected in glucose concentrations under fasting conditions (Figure 1D). HF mice also exhibited a significant glucose intolerance (Figure 1E,F; Figure D shows basal glucose values) compared with the control group. The replacement of the HF diet by a chow diet for 2 weeks reversed all these alterations (Figure 1B–F and Table 1).

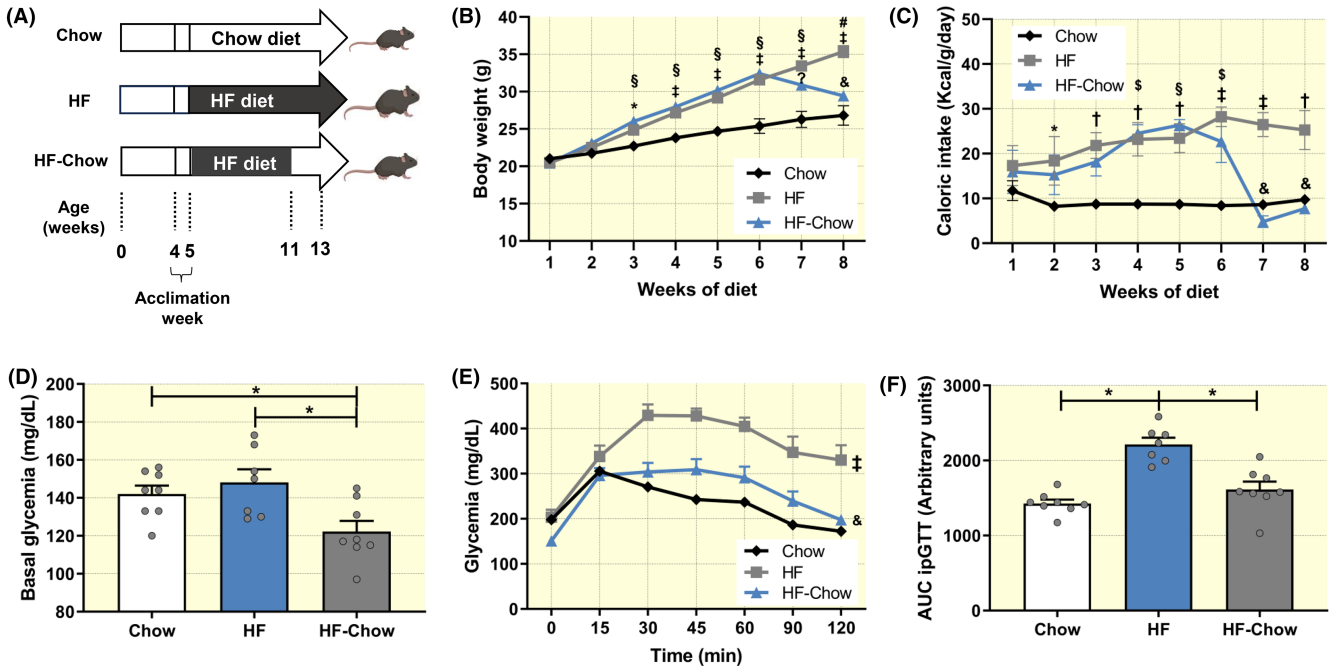


FIGURE 1 Animal model description. (A) Graphic scheme of the animal model design. Evolution of BW (B) and caloric intake (C). (D) Bars diagram shows basal glycemia measured 30 min before GTT. Glycemia (E) and AUC during the GTT (F). Data are represented as mean ± SEM of 7–8 determinations per group (Chow $n = 8$; HF $n = 7$; HF-Chow $n = 8$). $^{*}p < 0.05$, $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ HF vs. Chow; $^{\#}p < 0.05$, $^{\$}p < 0.01$ and $^{\S}p < 0.001$ HF-Chow vs. Chow; $^{?}p < 0.05$ and $^{\&}p < 0.001$ HF-Chow vs. HF (one-way ANOVA followed by Tukey's test or two-way ANOVA followed by Bonferroni's post hoc test, as appropriate).

TABLE 1 Effect of dietary treatment on body weight, adiposity, and biochemical parameters.

	Chow	HF	HF-Chow
Body weight (g)	26.8 ± 0.6	35.3 ± 0.9‡	28.5 ± 0.5$^{\&}$
PR-AT (mg/mm)	3.5 ± 0.5	26.4 ± 1.8‡	7.0 ± 0.7$^{\&}$
Mes-AT (mg/mm)	4.9 ± 0.6	18.9 ± 1.9‡	4.8 ± 0.3$^{\&}$
SC-AT (mg/mm)	13.2 ± 1.1	58.7 ± 1.0‡	20.1 ± 1.9$^{\&}$
PG-AT (mg/mm)	17.3 ± 10.6	88.6 ± 6.6‡	30.9 ± 3.0$^{\&}$
PAT-AT (mg/mm)	0.9 ± 0.21	1.6 ± 0. 2*	1.0 ± 0.1$^{?}$
BAT (mg/mm)	5.2 ± 0.4	11.2 ± 1.8†	5.9 ± 0.5$^{\sigma}$
Liver (mg/mm)	52.4 ± 1.5	55.3 ± 1.8	53.8 ± 1.6
Biochemical parameters			
Plasma TG (mg/dL)	101.1 ± 6.6	98.0 ± 5.3	92.8 ± 9.2
Plasma cholesterol (mg/dL)	116.4 ± 5.0	168.1 ± 7.0‡	118.5 ± 5.4$^{\&}$

Note: Biochemical parameters were assessed in non-fasted conditions. Tissue weights are expressed in mg per mm tibia length, which was not different between groups. $^{*}p < 0.05$, $^{\dagger}p < 0.01$ and $^{\ddagger}p < 0.001$ vs. Chow group; $^{?}p < 0.05$, $^{\sigma}p < 0.01$ and $^{\&}p < 0.001$ vs. HF group (one-way ANOVA). Data are expressed as mean ± SEM of 8 determinations per group. Perirenal (PR-AT), mesenteric (Mes-AT), periaortic (PA-AT), subcutaneous adipose tissue (SC-AT), perigonadal adipose tissue (PG-AT) and brown adipose tissue (BAT). Triglycerides (TG).

2.2 | HF feeding enhanced contractile responses due to defective NO bioavailability

As shown in Figure 2A, contractions to Phe (10^{-8} to 10^{-5} M) were significantly enhanced in HF mice compared to the Chow group, but unchanged when the chow diet was restored. Previously, we discarded that there were changes in the maximal contractile ability of the thoracic aorta in response to KCl (60 mM; Chow = 0.42 ± 0.02 g; HF = 0.44 ± 0.02 g; HF-Chow = 0.48 ± 0.02 g).

To determine the contribution of NO to vascular responses, we performed curves to Phe (10^{-8} to 10^{-5} M) in presence or

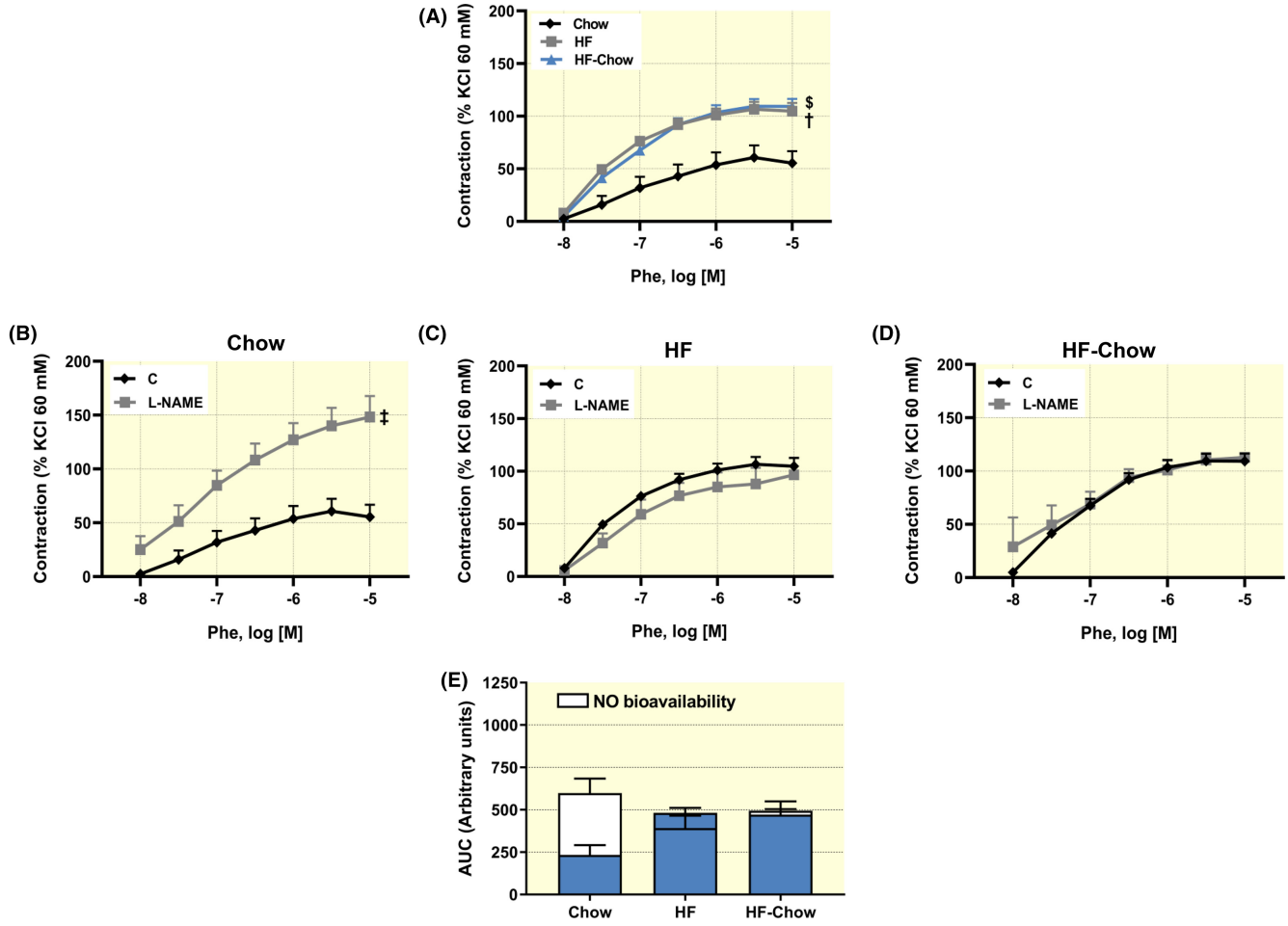


FIGURE 2 Contribution of NO to contractile responses. Cumulative concentration-response curves to phenylephrine (10^{-8} to 10^{-5} M) (A). $^{\dagger}p<0.01$ HF vs. control group and $^{\$}p<0.01$ HF-Chow vs. Chow (Two-way ANOVA; Bonferroni's comparison test). Data are represented as mean ± SEM of 6–7 determinations per group (Chow $n=6$; HF and HF-Chow $n=7$). (B–D) Cumulative concentration-response curves to phenylephrine (10^{-8} to 10^{-5} M) in aortic segments pre-incubated or not with L-NAME (B–D). $^{\ddagger}p<0.001$ L-NAME vs. control group (one-way ANOVA followed by Tukey's test or two-way ANOVA followed by Bonferroni's post hoc test, as appropriate). (E) AUC from cumulative concentration-response curves to phenylephrine (10^{-8} to 10^{-5} M) in aortic segments in absence (colored bar) or in presence (full histogram) of L-NAME. Data are represented as mean ± SEM of 6–7 determinations per group in C without L-NAME (Chow and HF $n=6$; HF-Chow $n=7$) and 3–5 determinations in segments preincubated with L-NAME (Chow and HF $n=5$; HF-Chow $n=3$).

absence of L-NAME (10^{-4}M). Whereas, the preincubation with L-NAME significantly increased contractions to Phe in Chow mice, it did not modify vascular responses in HF or HF-Chow mice (Figure 2B–D). These data, together with the differences in the AUC obtained in absence or presence of L-NAME, which indirectly reflects NO bioavailability (Figure 2E), indicate defective NO production in HF mice that was not reversed by short-term dietary intervention.

2.3 | The dietary intervention improved HF-feeding-derived endothelial dysfunction through the upregulation of basal AMPK

To assess the impact of HF feeding on endothelial function, we performed cumulative concentration-response curves to ACh (10^{-9} to 10^{-4}M). As shown in Figure 3A, HF mice exhibited a significant impairment in endothelial-dependent relaxation to ACh and a reduction of E_{max} compared with Chow animals (E_{max}Chow = 86.2 ± 2.4% vs. E_{max}HF = 64.6 ± 4.0%; $p<0.001$). Interestingly, we observed that endothelial function was significantly improved after dietary intervention (E_{max}HF-Chow = 76.8 ± 1.7%; $p<0.05$ vs. HF mice). In contrast, endothelium-independent relaxation elicited by SNP (10^{-12} to 10^{-5}M) was similar between all experimental groups (E_{max} Chow = 100.8 ± 1.9% vs. E_{max} HF = 113.2 ± 9.5% vs. E_{max} HF-Chow = 99.9 ± 5.7%), excluding alterations in the functionality of the smooth muscle cells.

To elucidate whether the increase in relaxant responses to ACh after dietary invention was linked to

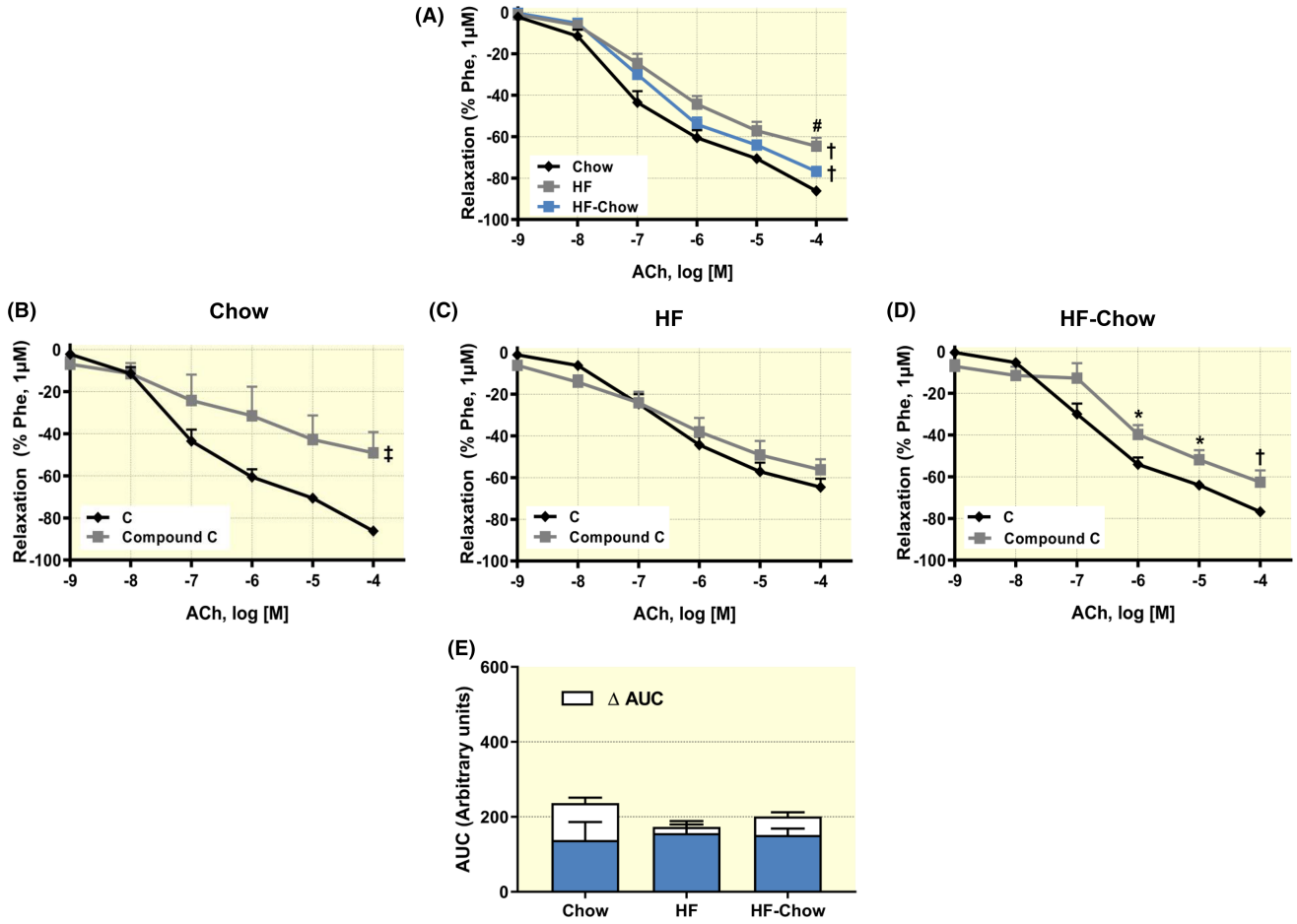


FIGURE 3 Contribution of AMPK to ACh-induced relaxant responses. Cumulative concentration-response curves to ACh (10^{-9} to 10^{-4} M). $^{\dagger}p<0.01$ HF vs. control group and $^{\#}p<0.05$ HF-Chow vs. Chow. (B–D) Cumulative concentration-response curves to ACh (10^{-9} to 10^{-4} M) in aortic segments from Chow (B) HF (C) and HF-Chow (D) animals pre-incubated or not with Compound C. (B) $^{\ddagger}p<0.001$ vs. control (Two-way ANOVA; Bonferroni's comparison test). (D) $^{*}p<0.05$ and $^{\dagger}p<0.01$ vs. C (Student's *t* test). (E) AUC of relaxant responses elicited by ACh, showing in white the AUC variation between curves in presence and absence of compound C. Data are expressed as mean ± SEM of $n=8$ determinations per group in C without Compound C and 4–7 determinations in segments preincubated with Compound C (Chow $n=5$; HF $n=7$; HF-Chow $n=4$).

AMPK activity, we analyzed relaxant responses to ACh (10^{-9} to 10^{-4} M) in presence of Compound C (10^{-5} M). As expected, we observed that ACh-induced relaxation was significantly reduced in presence of Compound C in Chow mice (Figure 3B) while this effect was not observed in HF mice (Figure 3C). These data indicate that HF feeding significantly compromises AMPK-mediated endothelial responses. However, in HF-Chow mice the presence of Compound C significantly reduced ACh-induced responses (Figure 3D), although to a smaller extent as compared with the Chow group. Moreover, as shown in Figure 3E, the difference in the AUC in absence or presence of Compound C (in white), which indirectly reflects basal AMPK activity, confirms that the dietary intervention increased, but did not normalized the contribution of AMPK to vascular responses to ACh.

2.4 | The dietary intervention restored AMPK activity

To elucidate whether the dietary intervention could increase AMPK activity, relaxant responses to AICAR were determined. As shown in Figure 4A, relaxation to AICAR (10^{-5} to 8.10^{-3} M) was significantly higher in HF-Chow arteries compared with the HF group (E_{max}HF-Chow = 83.9 ± 4.3%; vs. E_{max}HF = 56.8 ± 4.2%; $p<0.001$) and not significantly different from Chow mice (E_{max} = 83.0 ± 7.7%).

Recently, it was described that celecoxib induces vascular protection though the activation of the AMPK/CREB signaling pathway.[14] To confirm this hypothesis, we assessed relaxant responses to AICAR in arteries precontracted with Phe (10^{-6} M) and in the presence or absence of celecoxib (3×10^{-6} M). Intriguingly, the preincubation with celecoxib significantly reduced AICAR-induced relaxation in HF mice,

effect that was not observed in Chow or in HF-Chow mice (Figure 4B–D). These data, together with the differences in the AUC in the absence or presence of celecoxib (Figure 4E), indirectly suggest decreased AMPK activation in HF mice, that was completely reversed by the dietary intervention.

2.5 | HF mice exhibited a reduction in AMPK expression, CREB phosphorylation and the expression of antioxidant systems that was reversed by the dietary intervention

To better elucidate the mechanisms by which the dietary intervention was able to improve alterations induced by HF feeding on endothelial function and AMPK-mediated vascular responses, we analyzed the activation of the AMPK/CREB signaling pathway.

Although AMPK phosphorylation (Figure 5A) and CREB expression (Figure S1) were not modified in HF mice, they exhibited a significant reduction in both total AMPK expression (Figure 5B) and CREB phosphorylation (Figure 5C). In addition, a significant reduction of antioxidant systems including HO-1 and catalase was also detected in HF mice (Figure 5D,E). These alterations were reversed by the dietary intervention (Figure 5A–E and Figure S1), thus reaching protein levels similar to the Chow mice. No changes were observed in SOD-2 (Figure 5F) protein levels.

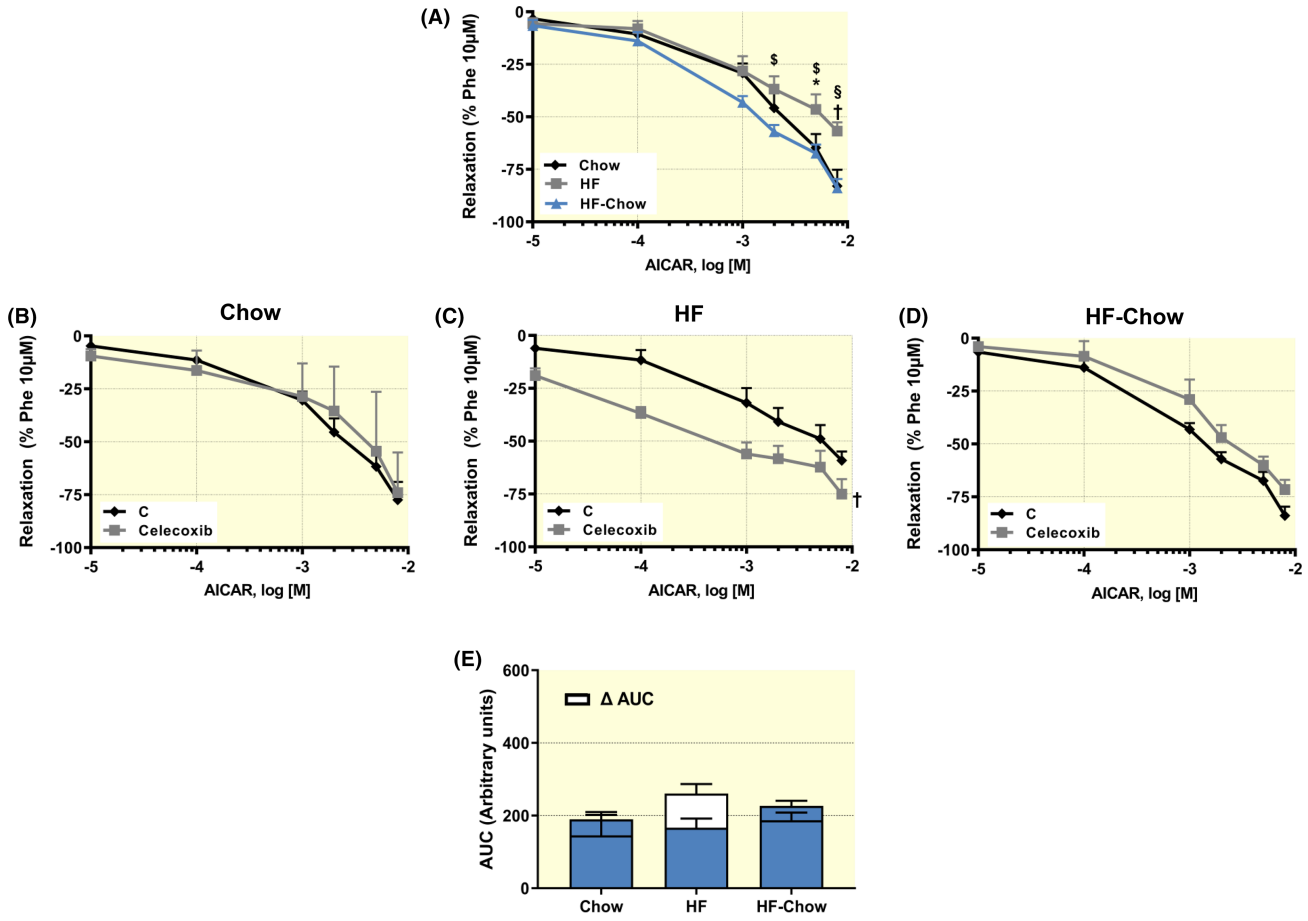
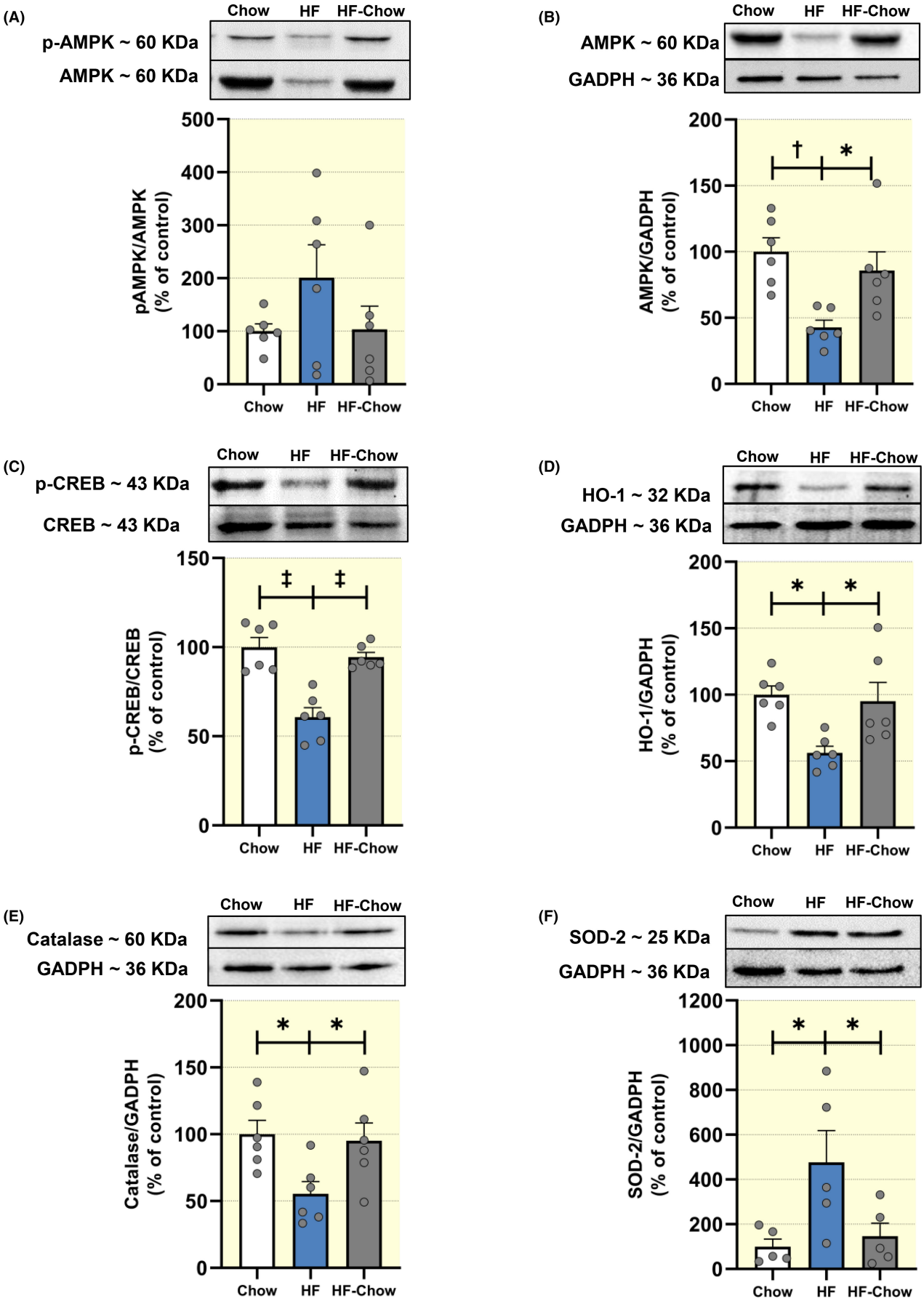


FIGURE 4 Contribution of COX-2 to AICAR-induced relaxant responses. Cumulative concentration-response curves to AICAR (10^{-5} to 10^{-2} M). (B–D) Cumulative concentration-response curves to AICAR (10^{-5} to 10^{-2} M) in aortic segments from Chow (B), HF (C) and HF-Chow (D) animals pre-incubated or not with Celecoxib. (E) AUC of relaxant responses elicited by AICAR, showing in white the AUC variation between curves in presence and absence of celecoxib. (A) $^{*}p<0.05$ and $^{\dagger}p<0.01$ HF vs. control group and $^{\$}p<0.01$ and $^{\S}p<0.001$ HF-Chow vs. Chow. (B–D) $^{\ddagger}p<0.001$ vs. control (Two-way ANOVA; Bonferroni's comparison test). Data are expressed as mean ± SEM of $n=7$ determinations per group in C without celecoxib and 3–4 determinations in segments preincubated with celecoxib (Chow $n=3$; HF $n=4$; HF-Chow $n=4$).

FIGURE 5 AMPK-mediated regulation of antioxidant systems. Representative immunoblots of p-AMPK/AMPK (A), AMPK/GAPDH (B), p-CREB/CREB (C), HO-1/GAPDH (D), catalase/GAPDH (E) and SOD-2/GAPDH (F) expression in thoracic aorta. Bars graph shows the result of densitometric analysis of immunoblots expressed as percentage of results obtained in Chow mice. Data are represented as mean ± SEM of $n=6$ determinations per group. $^{*}p<0.05$, $^{\dagger}p<0.01$ and $^{\ddagger}p<0.001$ (one-way ANOVA followed by Bonferroni's test).



Together, these data demonstrate that the dietary intervention restored the activation of the AMPK/CREB signaling pathway and antioxidant systems impaired by HF feeding, thus accounting for an improvement in endothelial function and AMPK-mediated vascular responses.

3 | DISCUSSION

Many studies have highlighted the importance of endothelial dysfunction and oxidative stress among the mechanisms responsible for obesity-related CVD.[7,15,16] In addition, AMPK, whose activation triggers NO release and thus, vascular protection, has shown to be deregulated in obesity contributing to these alterations.[7] Interestingly, CR, one of the most common strategies for the prevention and treatment of obesity-derived comorbidities, may reverse this effect.[8] However, we and others have recently evidenced the importance of the nutritional composition of the diet and not only the energy it provides in the development of vascular alterations.[4,5] Here, we demonstrate that a short-term dietary intervention based on the replacement of a HF diet by a standard healthy diet is a useful strategy for the prevention and/or treatment of obesity-derived vascular disorders and suggest a potential mechanism for these beneficial effects.

In the studies presented here, we used a mouse model of HF diet-induced obesity submitted to a healthy diet for only 2 weeks after 6 weeks of HF-feeding. It is important to note that studies of our group have previously revealed vascular alterations, including endothelial dysfunction and a defective NO release, in mice fed a HF diet for 6 weeks.[17–19] The main findings of this study are that restoring a healthy diet for a period as short as 2 weeks after HF diet intake was enough to reduce BW and improve metabolic parameters, endothelial function, and AMPK-derived vascular responses in the thoracic aorta, due to the activation of the AMPK/CREB signaling pathway.

Hypercaloric diets, including not only HF[7,16–19] but also high sucrose[20,21] and high fructose[22,23] diets, have all been shown to induce metabolic and vascular alterations. Several strategies, including moderate CR[24,25] and more recently, IF[26,27] or even time-restricted eating[28] have emerged as effective interventions for BW loss and improved health outcomes. However, the long-term efficacy of these interventions remains to be elucidated and further studies in this direction are required.[27] Herein, we demonstrate that the administration of a standard chow diet ad libitum for only 2 weeks after 6 weeks of HF diet exposure normalizes BW, adiposity, glucose and cholesterol levels, and glucose tolerance. Intriguingly, a recent study performed in a murine model of diet-induced obesity supports that switching to a sucrose-rich diet after western diet intake elicits weight loss and decreases obesity-induced metabolic complications and points out the potential of carbohydrates to treat obesity.[29] However, we have recently evidenced that the nature of the carbohydrates and lipids included in the diet, and not only the amount of these macronutrients, is crucial to maintain cardiometabolic health.[4] These results are aligned with the *2019 Position Statement from the American College of Cardiology/American Heart Association (ACC/AHA) and the European Society of Cardiology/European Atherosclerosis Society (ESC/EAS)* supporting healthy dietary patterns and includes the following recommendations: increased consumption of fiber-rich complex carbohydrates but limited intake of less healthy carbohydrate-rich foods, such as refined starches or sugars, a reduced consumption of saturated fat and a moderate intake of MUFA and PUFA, together with low-salt and moderate alcohol consumption, and the promotion of physical exercise.[5]

As expected, and according to previous results of our group and others,[17,18,30] HF mice exhibited impaired endothelial function and increased contractile responses to Phe associated to a decreased NO bioavailability. Interestingly, intake of the healthy diet significantly improved endothelial function although it was unable to normalize NO bioavailability and contractile responses, probably because of the short duration of the intervention. Other dietary interventions such as CR and IF have also been shown to improve endothelial function in different experimental conditions, including HF diet-induced obesity in mice, patients with metabolic syndrome, or aged mice among others, and by several mechanisms associated with NO availability.[3] However, only 2 weeks of severe CR have demonstrated to also trigger endothelial dysfunction and other cardiac alterations.[31] Similarly, severe IF interventions (more than 70% calorie intake reduction) are associated with important adverse effects, including fatigue, irritability, mood disorders, concentration difficulties, uncontrolled hyperphagia, etc. Moreover, long-term safety data of IF regimens are still missing.[3]

One of the major pathogenic factors proposed to be involved in the development of metabolic syndrome-related disorders is AMPK dysregulation. Indeed, several studies have revealed compromised AMPK activity in the vascular wall from both animal models of obesity and in humans.[6] In the present study, we also observed impaired AMPK-mediated relaxant responses to both Ach and AICAR. Interestingly, the intake of a healthy diet significantly enhanced AMPK-mediated vascular responses. These data are similar to previous results from our group showing that CR activates the endothelial AMPK-PI3K-Akt-eNOS pathway in a genetic model of obesity, the Zücker rat.[8] However, although we detected

a reduced NO bioavailability in HF and HF-Chow mice, relaxant responses to ACh nor to AICAR in presence of L-NAME could not be assessed. Therefore, we cannot discard that AMPK-meditated relaxation could be due to an increased NO release. Nevertheless, NO is not the only endothelium-dependent mechanism that might be activated by AMPK. Indeed, the role for PGI_2 in metformin-induced vasodilation on mesenteric vascular bed has also been described.[32] In addition, several strategies aimed at stimulating AMPK have been shown to elicit beneficial vascular effects, including lifestyle changes (i.e., physical activity or CR)[33] and pharmacological tools, such as metformin,[34] thiazolidindiones,[35] glucagon-like peptide-1 agonists,[36] etc. As a result, vascular AMPK has been recently proposed as a potential useful target for the management of obesity-related comorbidities.

In addition to the mechanism of direct NO release, an important role for CREB, a known AMPK target, for the maintenance of vascular homeostasis through the activation of anti-inflammatory and antioxidant enzymes, such as the HO-1, was recently proposed.[13] To shed some light on the role of the AMPK/CREB signaling pathway on the vascular alterations observed in our model, and since a recent study has revealed that treatment with celecoxib might directly activate this mechanism in endothelial cells,[14] we assessed the vascular responses to AICAR in the presence or absence of celecoxib. Interestingly, whereas preincubation with celecoxib significantly enhanced AMPK-mediated vascular relaxation in HF mice, this effect was not detected in Chow nor in HF-Chow mice, that indirectly suggests defective activation of the AMPK/CREB signaling pathway in obese mice that is reversed by a healthy diet. We also detected a significant reduction in AMPK protein expression and CREB phosphorylation in aortas from HF mice together with smaller amounts of both HO-1 and catalase, with these alterations also being reversed by the intake of the Chow diet. In this regard, AMPK has been pointed out as an inducer of HO-1 gene expression.[11] Moreover, several studies have revealed an important cytoprotective role of HO-1 in endothelial cells. Indeed, HO-1 not only exhibits antiapoptotic,[37] anti-inflammatory and angiogenic effects[11,38,39] but it has also shown to normalize endothelial cell function and blood pressure under several pathological circumstances.[39]

4 | MATERIALS AND METHODS

4.1 | Animal model design

Assays were carried out in 4-week-old male C57BL/6J mice bred in the animal facilities from Universidad CEU-San Pablo. Animals were housed under controlled light–dark cycles (12/12 h), temperature (22–24°C) and relative humidity (44%–55%) conditions and had access to food and water ad libitum. After 1 week of acclimation, animals were randomly assigned to a control (Chow; Teklad Rodent Diet 2918, 18% of kcal from fat; 3.1 kcal/g; Envigo, USA) or to a high-fat diet (HF; 58Y1, 62% of kcal from fat; 5.1 kcal/g; Test Diet, UK) for 8 weeks. The detailed composition of the diets has been included in Table S1. For the last 2 weeks, the HF diet was replaced by the chow diet in half of HF mice, thus generating 3 experimental groups; Chow, HF and HF-Chow (Figure 1A).

BW and food intake were monitored weekly. At the end of the dietary treatment, mice were weighed and euthanized by decapitation under inhalational anesthesia with isoflurane (5%). Blood was collected in EDTA-coated tubes, centrifuged at 800*g* for 10 min and plasma samples were stored at −80°C until used for biochemical analysis. Thoracic aortas were immediately dissected for both vascular function and western blotting studies. Fat depots, including perirenal (PR-AT), mesenteric (Mes-AT), periaortic (PAT-AT), subcutaneous adipose tissue (SC-AT), perigonadal (PG-AT), and brown adipose tissue (BAT), as well as the liver were weighed and normalized by the tibial length.

All experiments were performed in accordance with the European Union Laboratory Animal Care Rules (86/609/ECC directive) and approved by the Ethical Committee of the San Pablo CEU University and the Animal Protection Area of the Comunidad Autónoma de Madrid (PROEX 061/16).

4.2 | Assessment of biochemical parameters

Triglyceride and cholesterol levels were analyzed using the GPO (Biolabo) and the CHOD-POD (Spinreact) methods, respectively.

4.3 | Glucose tolerance determination

One week before sacrifice, an intraperitoneal glucose tolerance test (ipGTT) was performed to determine glucose tolerance. Briefly, animals were fasted for 6 h before the glucose load (ip. bolus of 1 g/Kg at time 0). Glycemia was measured in blood samples from the tail vein of conscious mice right before glucose injection and 15, 30, 45, 60, 90, and 120 min after injection with an Accu-Chek Aviva glucometer (Roche Diagnostics).

4.4 | Vascular function in the thoracic aorta artery

Thoracic aortas, previously dissected and deprived of perivascular adipose tissue, were cut into 2–3-mm length rings. Vascular function was analyzed by isometric tension recording in arterial segments, as previously described.[40]

Arterial integrity was determined with potassium chloride (KCl, 60 mM) and contractile capacity was analyzed with phenylephrine (Phe, 10^{-8} to 10^{-5} M). Endothelial integrity and endothelial-independent relaxation were evaluated with acetylcholine (ACh, 10^{-9} to 10^{-4} M) and sodium nitroprusside (SNP, 10^{-12} to 10^{-5} M), respectively, in rings pre-contracted with a submaximal concentration of Phe, 10^{-6} M. Basal AMPK activity was analyzed by comparing the relaxation to ACh (10^{-9} to 10^{-4} M) in the presence or absence of Compound C (10^{-5} M, 20 min; AMPK inhibitor). In another set of experiments, cumulative curves to 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, 10^{-5} to 8×10^{-3} M; AMPK activator) were performed in segments pre-contracted with Phe, 10^{-6} M. In some experiments, aortic rings were preincubated with NG-nitro-L-arginine methyl ester (L-NAME, unspecific NOS inhibitor, 10^{-4} M) or celecoxib (specific cyclo-oxygenase inhibitor, 3×10^{-6} M) for 20 min before Phe or AICAR administration.

4.5 | Chemicals

ACh was dissolved in saline and Phe and SNP in 0.01% ascorbic acid/saline. Compound C, and celecoxib were prepared in DMSO and AICAR and L-NAME in water. All reagents were obtained from Sigma-Aldrich (Spain) except for AICAR that was from LGC Standards, S.L.U (Spain).

4.6 | Western blotting

Western blotting experiments were performed in thoracic aorta as previously described.[41] Briefly, equivalent amounts of proteins (20 μg) were loaded in 10%–15% polyacrylamide-SDS gels (SDS-PAGE), as appropriate and transferred to PVDF membranes (BioRad) using the Trans-Blot®Turbo™ system (Bio-Rad).

Primary antibodies against AMPKα and p-AMPKα$^{(Thr172)}$ (1:500 final dilution; Cell Signaling Technology), CREB and p-CREB$^{(Ser\ 133)}$ (1:400 and 1:100 final dilution, respectively; Cell Signaling Technology), HO-1 (1:250 final dilution, Santa Cruz biotechnology), catalase (1:2000 final dilution, Sigma-Aldrich), Mn-SOD (1:250 final dilution, Santa Cruz biotechnology), and GAPDH (1:5000 final dilution, Sigma-Aldrich) were incubated overnight at 4°C. After washing, an appropriate secondary antibody (anti-rabbit or anti-mouse IgG peroxidase conjugated) was applied for 1 h. Blots were incubated in a commercial enhanced chemiluminescence reagent ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Germany) and analyzed using the software Imaging System (Molecular imager® ChemiDoc™ XRS+, BIO-RAD). Expression values of AMPK, HO-1, catalase, and Mn-SOD were normalized with GAPDH to account for variations in gel loading. Values for p-AMPKα and p-CREB were normalized with AMPKα and CREB values, respectively.

4.7 | Data analysis

Contractile responses to Phe are expressed as the percentage of the maximal contractile response to KCl. Relaxant responses are expressed as the percentage of the previous contraction elicited by Phe. The area under the curve (AUC) was calculated from each individual concentration-response curve plot (GRAPHPAD Software, California, United States). The maximum response (E_{max}) was calculated by non-linear regression analysis of each individual concentration-response curve.

All data are expressed as mean ± SEM and n denotes the number of animals used in each experiment. Outliers were identified with the Rout method, using a $Q=1\%$. The normal distribution of each variable was verified using both the Shapiro–Wilk and the Kolmogorov–Smirnov tests. A value of $p<0.05$ was considered statistically significant. Significant differences were assessed using Student's t test, a one-way analysis of the variance test (1-ANOVA) followed by Tukey's test or two-way ANOVA (2-ANOVA) followed by Bonferroni's post hoc test, as appropriate. All statistical analyses were performed using GRAPHPAD PRISM 8 software.

5 | CONCLUSIONS

These data demonstrate the efficacy of healthy dietary habits to improve obesity-derived endothelial dysfunction and AMPK-mediated vascular responses, at least partially due to the activation of the AMPK/CREB/HO-1 signaling pathway and support the potential of therapeutic strategies that trigger AMPK induction for the treatment of obesity-related vascular alterations.

AUTHOR CONTRIBUTIONS

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ACKNOWLEDGMENTS

We thank J.M. Garrido, J. Bravo, I. Bordallo, and M. Fajardo for skillful animal care and technical support.

FUNDING INFORMATION

This work was supported by Fundación Universitaria San Pablo CEU – Santander; Ministerio de Economía y Competitividad (BFU2017-82565-C2-2-R); and Grupos Universidad Complutense de Madrid (GR-921641).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: González-Blázquez R, Gil-Ortega M, Alcalá M, et al. Short-term dietary intervention improves endothelial dysfunction induced by high-fat feeding in mice through upregulation of the AMPK-CREB signaling pathway. *Acta Physiol*. 2023;239:e14023. doi:10.1111/apha.14023