# Absence of endothelial dysfunction, but augmented $\alpha_1$-adrenergic contractions in renal arteries of aged obese mice independently of nitric oxide production and constitutive activation of endothelial adenosine monophosphate-activated protein kinase

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## Abstract

***Background:*** Aging and obesity favor vascular dysfunction. Endothelial activation of adenosine monophosphate-activated protein kinase (AMPK) has protective effects in diabetes. ***Methods:*** Mice with constitutive endothelial activation of AMPK (CA-AMPK) were given high fat diet to induce obesity or kept on standard chow as lean controls for up to two years. A subset of obese animals was changed to standard chow after thirty weeks of high fat feeding. Endothelium-dependent and –independent responses were examined by isometric tension recording. ***Results and Conclusion:*** Endothelium-dependent nitric oxide (NO)- and hyperpolarization-mediated relaxations were preserved and similar between aortic or renal arterial preparations of lean and obese CA-AMPK mice and their wild type littermates. Despite comparable release of vasoconstrictor prostanoids, cyclooxygenase-dependent contractions were aggravated during NO synthase inhibition in carotid arterial rings of obese CA-AMPK mice. Contractions to the $\alpha_1$-adrenoceptor agonist phenylephrine were augmented in renal arteries of obese animals, a phenomenon reversible by weight loss and genotype-independent. These data indicate a higher $\alpha_1$-adrenergic reactivity in renal arteries of aged mice with obesity. The current results highlight the potential of weight loss to alleviate vascular dysfunction. However, endothelial activation of the AMPK pathway in obesity may not be sufficient to prevent vascular dysfunction without lifestyle changes.

**Key Words:**

Acetylcholine; EDCF; EDH; Endothelium; lifestyle; Phenylephrine; TP receptors; weight loss.

## Introduction

Obesity is an independent cardiovascular risk factor (Hubert *et al.*, 1983), threatens vascular health with aging (Brunner *et al.*, 2015), and has been associated with cyclooxygenase-dependent vascular dysfunction in rodent and human arteries in the absence of relevant increases in arterial blood pressure or fasting glucose levels (Traupe *et al.*, 2002; Farb *et al.*, 2014; Baretella *et al.*, 2017). However, as regards life expectancy in both humans and mice (Rowlatt *et al.*, 1976), these and most other functional studies were carried out in arteries of relatively young subjects. Aging is a non-modifiable vascular risk factor known to promote endothelium- and cyclooxygenase-dependent contractions in rodent arteries while endothelium-dependent relaxations are preserved (Koga *et al.*, 1989).

The adenosine monophosphate-activated protein kinase (AMPK) pathway exerts protective effects on the endothelium favoring endothelial nitric oxide (NO) production (Fisslthaler & Fleming, 2009). AMPK is activated by the anti-diabetic drug metformin (Zhou *et al.*, 2001) or the Chinese herb berberine (Wang *et al.*, 2009), and constitutive activation of this pathway in the endothelium (CA-AMPK) alleviates endothelial dysfunction in type 1 diabetic mice (Li *et al.*, 2012). Furthermore, sustained activation of AMPK is ameliorating vascular dysfunction in aging mice independent of nitric oxide (Lesniewski *et al.*, 2012). The present experiments were designed to test the hypothesis that constitutive endothelial activation of AMPK (CA-AMPK) protects against vascular dysfunction in arteries of both lean and obese aged mice in terms of reduced endothelium-dependent relaxations in large and smaller arteries as well as augmented endothelium-dependent and direct activation of vascular smooth muscle. In addition, some initially high fat-fed animals with or without CA-AMPK were changed from high fat diet to standard chow following the development of obesity to evaluate the effect of weight loss on vascular function. Longevity was compared between

lean CA-AMPK mice and their wild type littermates to evaluate whether or not constitutive activation of this pathway in the endothelium has implications on endothelium-dependent and indepented vascular responsiveness and life expectancy.

## Materials and methods

### Animal studies

Male mice with a constitutively active AMPK (CA-AMPK) in endothelial cells (Li *et al.*, 2012) were mated with female C57BL/6N wild type (WT) mice and their offspring genotyped to identify CA-AMPK mice and their WT littermates. Therefore, specific primers for the construct [consisting of the vascular endothelial (VE)-cadherin promoter (forward: 5'-ACA AAG CTC GGC CCT GGA CAG-3') and AMPK (reverse: 5'-GAT CTC TCT GCG GAT TTT CCC G-3')] were used showing a band of 250 base pair size following genotyping as described (Li *et al.*, 2012), confirming the presence of this construct (Supplemental Figure 1). Following weaning at the age of four weeks, male CA-AMPK mice (Li *et al.*, 2012) and their wild type (WT) littermates on the same C57BL/6N background were randomized to standard chow (13% kcal from fat, D5053, Lab Diet, Purina Mills, Richmond, IN, USA) or high fat diet (41% kcal from fat, D12079B, Research Diets Inc, New Brunswick, NJ, USA). After thirty weeks, a subset of animals from either genotype on high fat diet was changed to standard chow (*obese to lean*), while the other mice remained on the initial diet for a total of twenty or more months. The animals were housed at constant temperature under a twelve hours light-dark cycle with free access to food and water, and their body weight was monitored weekly individually. At the end of the study, systolic arterial blood pressure was measured by the non-invasive tail-cuff method (BP-2000 Blood Pressure Analysis System, Visitech Systems Inc., Raleigh, NC, USA) in conscious and trained mice (Krege *et al.*, 1995). On the day of final experiments, the over-night fasted animals were anaesthetized with pentobarbital sodium (70 mg/mL/kg; Alfasan, Woerden, The Netherlands) and sacrificed by exsanguination via cardiac puncture. Blood was collected into chilled EDTA tubes, centrifuged at 2000 rpm at 4° C for twenty minutes, and 100 µL plasma aliquots were stored at -80° C. Vascular tissues (carotid arteries, thoracic aortae, main- and segmental renal

arteries) were harvested for isometric tension recording experiments and prostanoid levels in solution samples from some of these experiments were determined by ELISA as described (Baretella *et al.*, 2014; Baretella *et al.*, 2017). All procedures were approved by the institutional Committee on the Use of Live Animals for Teaching and Research (CULATR) of the University of Hong Kong in line with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals issued by the U.S. Institute of Laboratory Animal Research (ILAR, Eighth Edition, 2011).

**Experimental protocols for functional studies**

To ensure proper comparisons between preparations in the performed longitudinal study, each arterial ring was put individually at its optimal point of tension by stimulations with an equimolar high $K^+$ solution (60 mmol/L potassium chloride, with equimolar substitution of sodium by potassium) following each increase in passive tension. Thereby, the final optimal passive resting tension was between 20 to 25 mN for aortic rings, 15 to 18 mN for carotid arterial preparations, and 3 or 4 mN for segmental or main renal arteries, respectively. The increase in tension to high $K^+$ at the optimal point of this length-tension curve was used as reference contraction. There no significant differences upon exposure to high $K^+$ depolarizing solution between preparations of the different groups compared, except for a reduction in response in the thoracic aortae of both wild-type and CAMPK mice and an increased contraction in nain renal arteries of wild-type *obese to lean* animals(Supplementary Table 1). Experiments were performed on untreated rings or after incubation (thirty minutes) with vehicle or with the nitric oxide synthase inhibitor $N^{\omega}$-nitro-$_L$-arginine methyl ester (L-NAME, 3 x $10^{-4}$ mol/L) and/or the non-selective cyclooxygenase inhibitor indomethacin ($10^{-6}$ mol/L) prior to exposure to vasoactive agents. Adjacent thoracic aortic rings in the absence or presence of L-NAME, were exposed to increasing concentrations of the $\alpha_1$-adrenergic agonist

phenylephrine ($10^{-10}$ to $10^{-4}$ mol/L). Subsequently, relaxations to acetylcholine ($10^{-10}$ to $10^{-4}$ mol/L) or sodium nitroprusside ($10^{-11}$ to $10^{-6}$ mol/L; in the presence of L-NAME) were obtained in phenylephrine-contracted (70 to 100% of the reference response to high $K^+$) aortic preparations. Full concentration-contraction curves were obtained in quiescent carotid artery rings to acetylcholine ($10^{-10}$ to $10^{-4}$ mol/L) in the presence of L-NAME to optimize prostanoid-mediated responses (Auch-Schwelk *et al.*, 1992; Tang *et al.*, 2005a; Zhou *et al.*, 2005). Contractions of main renal arteries to phenylephrine were obtained in the absence and presence of L-NAME. Following repeated successive wash-outs, contractions were obtained to the TP receptor agonist U46619 ($10^{-11}$ to 3 x $10^{-6}$ mol/L) in aortic-, carotid-, and renal arterial rings. These experiments were performed in the presence of L-NAME and additionally that of indomethacin [to prevent the generation of endogenous prostanoids upon activation of endothelial TP receptors (Hanasaki *et al.*, 1988; Sung *et al.*, 1989)]. Segmental renal artery rings were contracted with phenylephrine in the presence of indomethacin only, indomethacin plus L-NAME, or indomethacin, L-NAME, and apamin plus charybdotoxin (both $10^{-7}$ mol/L) (Edwards *et al.*, 1998) to evaluate nitric oxide- and/or hyperpolarization-mediated relaxations to the endothelium-dependent agonist acetylcholine in this preparation.

**Prostanoid measurements**

Aliquots of 200 µL of myograph chamber solution were collected after exposure of carotid arterial rings to the final concentration ($10^{-4}$ mol/L) of acetylcholine. The aliquots were stored at -80° C for later determination of vasoactive prostanoids (Gluais *et al.*, 2005; Baretella *et al.*, 2014; Baretella *et al.*, 2017). Concentrations of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane $B_2$ as the stable metabolites of prostacyclin and thromboxane $A_2$, respectively, as well as that of prostaglandin $E_2$ were determined in undiluted aliquots using commercially available ELISA kits (Cayman Chemical Company, Ann Arbor, MI, USA) according to the

manufacturer's instructions.

**Drugs**

Acetylcholine chloride, apamin, charybdotoxin, indomethacin, L-NAME, and sodium nitroprusside dihydrate were obtained from Sigma-Aldrich (St. Louis, MO, USA); 9,11-Dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619) was from Enzo Life Sciences (Farmingdale, NY, USA). All drugs were dissolved in distilled water except indomethacin and U46619, which were prepared in sodium carbonate ($10^{-3}$ mol/L stock) and ethanol ($10^{-2}$ mol/L stock), respectively. Final concentrations were below 0.1% for both sodium carbonate and ethanol. Concentrations are given as final molar concentration in the myograph solution.

**Plasma levels of metabolic parameters**

Fasting plasma glucose levels were measured at sacrifice with a portable glucometer (Roche Diagnostics, Mannheim, Germany) using the enzymatic glucose oxidase method. Insulin and adiponectin were assayed with the *in-house* high sensitive mouse insulin (#32270) and mouse adiponectin (#32010) immunoassay kits, respectively (Antibody and Immunoassay Services AIS at The University of Hong Kong). Colorimetric assays were used to quantify total cholesterol (#1010), and triglycerides (#2100) (Stanbio Laboratory, Boerne, TX, USA).

**Calculations and statistical analysis**

Results are shown as means ± standard error of the mean (SEM), whereby *n* equals the number of mice per group. Contractions are expressed as percent of the reference contraction to high $K^+$ (60 mmol/L potassium chloride with equimolar substitution of sodium by potassium) and relaxations as percentage of contractions to phenylephrine. Distribution

normality was tested with the D'Agostino-Pearson test (GraphPad Software, San Diego, CA, USA). Concentrations causing half maximal responses ($EC_{50}$) were calculated and compared using nonlinear regression and expressed as negative logarithms ($pD_2$ values). The calculated maximal responses ($E_{max}$) are presented as percentages of the reference contraction. One-way ANOVA or the Kruskal-Wallis test followed by Bonferroni or Dunns analysis, respectively, were used to analyze statistical differences between unpaired groups as appropriate. Two-way ANOVA with repeated measurements were used for multiple comparisons of concentration-response curves, followed by Bonferroni *post hoc* analysis where appropriate. Student's *t*-test was used for direct comparisons of samples showing a normal distribution, while for non-parametric samples the Mann-Whitney *U* test was applied. *P* values lower than 0.05 were considered to indicate differences of statistical significance.

## Results

### Growth curves, cardiometabolic parameters, and life expectancy

Mice fed a high fat diet grew faster and were finally heavier than their counterparts kept on standard chow, and the animals which were obese after thirty weeks of high fat feeding rapidly lost body weight when the diet was changed to standard chow reversing towards control body weights as observed in their lean counterparts (Fig. 1 and Table 1). These effects of dietary treatments were similar between CA-AMPK and wild type (WT) mice. Life expectancy was slightly but not significantly augmented by constitutive activation of the AMPK pathway in endothelial cells (Supplemental Fig. 2). Weight loss by a change from high fat diet to standard chow (*obese to lean*) also reversed plasma cholesterol levels back to normal, while triglycerides and adiponectin levels were not significantly different between lean and obese mice (Table 1). Irrespective of constitutively active AMPK in endothelial cells, insulin levels were significantly higher with obesity, but all animals remained normoglycemic (Table 1). Systolic arterial blood pressures were in the normotensive range in all animals (Table 1). These findings demonstrate that, under the used experimental conditions, obesity was achieved without the occurrence of concomitant metabolic syndrome and/or diabetes.

### *Endothelium-dependent relaxations in the aorta*

The contracted rodent thoracic aorta exhibits both nitric oxide-mediated relaxations but also prostanoid-mediated contractions to higher concentrations of the endothelium-dependent muscarinic agonist acetylcholine (Lüscher & Vanhoutte, 1986; Traupe *et al.*, 2002). Contractions to phenylephrine ($10^{-10}$ to $10^{-4}$ mol/L) were similar in thoracic aortic rings from aged WT and CA-AMPK mice of the lean ($E_{max}$ 67±3% and 73±2% high $K^+$), *obese to lean* ($E_{max}$ 102±4% and 92±8% high $K^+$), or obese ($E_{max}$ 89±7% and 102±5% high $K^+$) phenotype.

Acetylcholine ($10^{-10}$ to $10^{-4}$ mol/L) relaxed these $\alpha_1$-adrenergically contracted preparations comparably across groups at lower concentrations, and higher concentrations caused similar secondary increases in tension in aortic rings from lean and obese mice of both genotypes (Fig. 2a). In line with the nitric oxide (NO)-mediated relaxations to acetylcholine, responses of the vascular smooth muscle to the NO donor sodium nitroprusside ($10^{-11}$ to $10^{-5}$ mol/L) [in the presence of 3 x $10^{-4}$ mol/L L-NAME] did not differ between aortic preparations of the different groups (Fig. 2b). Likewise, contractions upon smooth muscle TP receptor activation by U46619 ($10^{-11}$ to 3 x $10^{-6}$ mol/L) were similar between groups (Table 2). These results demonstrate the similar release and effect of NO in relaxing aortic preparations from lean and obese mice with or without constitutive activation of the AMPK pathway in the endothelium. Furthermore, contractions of vascular smooth muscle upon activation of adrenergic- and thromboxane-prostanoid receptors are comparable irrespective of such constitutive endothelial AMPK overexpression .

***Endothelium-dependent relaxations in segmental renal arteries***

All experiments in segmental renal artery rings were carried out in the presence of indomethacin ($10^{-6}$ mol/L) to exclude any effects of prostaglandins on vascular responses upon activation of muscarinic receptors. During similar contractions to phenylephrine, the endothelium-dependent agonist acetylcholine induced relaxations that were significantly enhanced in segmental renal arteries of lean but not obese CA-AMPK mice compared to corresponding preparations from WT littermates (Fig. 3a). By contrast, the propensity to relax to the muscarinic agonist was comparable and no longer improved by the CA-AMPK genotype irrespective of the phenotype in parallel experiments with segmental renal arteries incubated with L-NAME (Fig. 3b) indicating a NO-mediated response to be required for the improvement through CA-AMPK in these arteries. The responses to acetylcholine in

segmental renal arteries were almost abolished in the presence of apamin plus charybdotoxin (both $10^{-7}$ mol/L) (Edwards *et al.*, 1998) in addition to L-NAME (Fig. 3c) highlighting the contribution of endothelium-dependent hyperpolarization in relaxing these preparations upon exposure to increasing concentrations of the muscarinic agonist.

***Endothelium-dependent contractions in carotid arteries***

Quiescent murine *carotid* arteries allow investigating best prostanoid-mediated contractions in response to acetylcholine (Zhou *et al.*, 2005; Baretella *et al.*, 2014). Therefore, these prostanoid-mediated [sensitive to non-selective cyclooxygenase inhibition with $10^{-6}$ mol/L indomethacin (Zhou *et al.*, 2005)] contractions were studied in quiescent rings of carotid arteries. To optimize the response (Auch-Schwelk *et al.*, 1992; Tang *et al.*, 2005a; Zhou *et al.*, 2005), these experiments were conducted in preparations incubated with L-NAME. Acetylcholine induced comparable contractions in carotid rings of lean, *obese to lean*, and obese WT animals, while only in those of obese CA-AMPK mice these cyclooxygenase-dependent responses were augmented significantly (Fig. 4a). In the presence of indomethacin, contractions to acetylcholine were abolished in carotid arterial preparations of lean animals and obese WT mice only, while increases in tension still were recorded in corresponding rings from CA-AMPK littermates particularly following prolonged high fat feeding (Fig. 4b). The contractions to acetylcholine were paralleled by the release of 6-keto-prostaglandin $F_{1\alpha}$, and, to a lesser extent of prostaglandin $E_2$, but not of thromboxane $B_2$ (the levels of which were at the lower limit of the assay); the increases in prostanoid release were comparable among groups (Fig. 4c). Indomethacin significantly reduced in all groups the production of prostacyclin compared to control experiments (Fig. 4c) but that of prostaglandin $E_2$ only in arteries from WT mice on standard chow (Fig. 4c).

***TP receptor activation***

All experiments with the full TP receptor agonist U46619 ($10^{-11}$ to 3 x $10^{-6}$ mol/L) were performed in the presence of L-NAME and indomethacin, to prevent endogenous generation of nitric oxide and prostanoids, respectively. As in the thoracic aorta, carotid arterial rings of obese WT mice contractions were not increased upon TP receptor activation, but had even lower $pD_2$ values than in preparations of lean controls (Table 2). Corresponding preparations from CA-AMPK mice were slightly but significantly more sensitive to U46619 than carotid arterial rings from their lean or obese WT littermates (Fig. 5 and Table 2). By contrast, there were no significant differences in the response to the TP receptor agonist between main renal arteries of the respective groups (Table 2).

***Alpha$_1$-adrenergic activation in main renal arteries***

Contractions to the $\alpha_1$-adrenoceptor agonist phenylephrine ($10^{-10}$ to 3 x $10^{-4}$ mol/L) were comparable between genotypes, but the responses were shifted significantly to the left by obesity in main renal arteries of both aged WT and CA-AMPK mice (Fig. 6a). Change from high fat diet after thirty weeks to standard chow in *obese to lean* animals lead to similar responses as in preparations from lean controls being significantly to the right of those from aged obese mice (Fig. 6a). These changes were apparent also in corresponding experiments conducted in the presence of L-NAME, which similarly facilitated contractions to phenylephrine in main renal arteries from all groups (Fig. 6b).

## Discussion

In the present study the effect of obesity on vascular function was studied under stringent comparative conditions in the context of aging and constitutive activation of adenosine monophosphate-activated protein kinase (CA-AMPK) in endothelial cells. The experiments revealed no impairments in terms of endothelium-dependent relaxations and neither augmented endothelium-dependent contractions nor increased responsiveness to TP receptor activation of vascular smooth muscle cells, but augmented $\alpha_1$-adrenergic contractions to phenylephrine in renal arteries from obese mice in the absence and presence of nitric oxide synthase inhibition. This augmentation with obesity was absent in previously obese animals changed from a high fat diet to standard chow highlighting the potential of weight loss in the prevention of vascular dysfunction. By contrast, CA-AMPK had no promising effects on vascular function in obesity *per se*.

*A model of sustained obesity*

Vascular responses between lean and obese mice were examined in animals of a relatively high age compared to most research undertaken with diet-induced obesity usually limited to about thirty weeks of high fat feeding (Barton *et al.*, 2000; Traupe *et al.*, 2002; Belin de Chantemèle *et al.*, 2011; Baretella *et al.*, 2017). If mice are started on high fat diet after weaning around four weeks of age, they will be about nine months old at sacrifice, which is only one quarter of their three years maximal life expectancy (Rowlatt *et al.*, 1976), as confirmed in the lean animals of the present study. Therefore, mice up to two years old, as investigated here, appear to be a reasonable animal model to determine the effects of vascular aging, since older C57BL/6 mice have an increased tumor incidence preventing the majority of reaching the maximal life expectancy (Rowlatt *et al.*, 1976).

The normal arterial blood pressure repeatedly observed in mice with diet-induced obesity of moderate duration (Barton *et al.*, 2000; Belin de Chantemèle *et al.*, 2011; Baretella *et al.*,

2017) was preserved in the aged obese animals of the present study as well as their response to insulin (to judge from the normoglycaemic fasting glucose levels). Thus, as stated above, the present study provides a model with sustained obesity as an independent modifiable cardiovascular risk factor (Hubert *et al.*, 1983) without further apparent metabolic alterations or changes in systolic arterial blood pressure in the context of the non-modifiable aging condition.

*Endothelial function and vascular dysfunction in aged obese mice*

Endothelial dysfunction in terms of attenuated endothelium-dependent nitric oxide-mediated relaxations (Vanhoutte *et al.*, 2016) was absent in the present study in aged wild type animals on high fat diet as aortic relaxations to acetylcholine and the nitric oxide donor sodium nitroprusside were preserved compared to lean controls on standard chow. These findings are in line with the normal responses to both endogenous and exogenous nitric oxide in aortic preparations of obese and diabetic *ob/ob* mice (Mundy *et al.*, 2007) and in db/db mice with mutated toll-like receptor 4 (Liang *et al.*, 2013). They demonstrate that, provided obesity is the sole risk factor introduced, despite further aging and continued obesity, relaxations to acetylcholine in this conduit artery can be maintained at the normal level observed at a younger stage [thirty weeks of high fat feeding (Traupe *et al.*, 2002; Baretella *et al.*, 2017)] as it is also the case in aged rats (Koga *et al.*, 1989).

As originally described for aortic rings of the spontaneously hypertensive rat (Lüscher & Vanhoutte, 1986), and obese rather than lean mice (Traupe *et al.*, 2002), there was a secondary increase in tension in response to higher concentrations of acetylcholine in corresponding aortic preparations of aged lean and obese animals. By contrast to a previous study with mice after thirty weeks diet-induced obesity (Traupe *et al.*, 2002) and to the findings in aged rats (Koga *et al.*, 1989), contractions to higher concentrations of the muscarinic agonist were rather small and not augmented in aortic rings of aged obese compared to those of lean mice. The similarity in this TP receptor-dependent response

(Traupe *et al.*, 2002; Zhou *et al.*, 2005) between preparations of aged lean and obese animals is paralleled by a similar sensitivity in aortic rings of mice from all groups to full activation of the receptor by U46619. Endothelium-dependent prostanoid-mediated contractions in response to acetylcholine, as examined in quiescent rings of carotid arteries (Zhou *et al.*, 2005), were not augmented in corresponding preparations of aged obese compared to lean wild type mice. In this artery, the potency of the full TP receptor agonist U46619 was even decreased in wild type control animals on high fat diet compared to those fed standard chow. Taken in conjunction, the present findings demonstrate that obese C57BL/6 wild type mice do not develop endothelial dysfunction even after prolonged high fat feeding to judge from the relaxations to acetylcholine in rings of the thoracic aorta and of segmental renal arteries, which were similar in preparations of lean and obese animals. Moreover, endothelium-dependent prostanoid-mediated contractions were not augmented by obesity in the thoracic aorta or in carotid arteries of aged mice. This finding combined with the unchanged TP receptor responsiveness of the vascular smooth muscle also in renal arteries of aged obese mice even suggests the absence of any vascular dysfunction in these animals. However, the major positive finding of the present study is that unlike in aortic rings, contractions to phenylephrine in main renal arteries were facilitated by obesity.

*Effects of CA-AMPK on vascular responses*

Mice with a constitutively active adenosine monophosphate-activated protein kinase (CA-AMPK) in their endothelial cells (Li *et al.*, 2012) have been generated as a model to investigate continuous endothelial activation of this enzyme mimicking the beneficial effects of the adipose tissue-derived hormone adiponectin triggering this pathway (Zhu *et al.*, 2008; Xu & Vanhoutte, 2012) and as achieved pharmacologically with the anti-diabetic drug metformin (Zhou *et al.*, 2001; Davis *et al.*, 2006), or the Chinese herb berberine (Wang *et al.*, 2009). Endothelial function was indeed improved in these animals' arteries with type 1

diabetes and following vascular injury (Li *et al.*, 2012). By contrast, in the present study of aged animals with diet-induced obesity only, there was hardly any endothelial dysfunction as endothelium-dependent relaxations were largely preserved and endothelium-dependent contractions not augmented in obese wild type mice compared to lean control animals. The activity of nitric oxide synthase appears to be crucial for endothelial function in mice with a constitutively active AMPK pathway (Fisslthaler & Fleming, 2009; Li *et al.*, 2012). Hence, in the absence of L-NAME, relaxations to acetylcholine were enhanced in renal artery preparations of lean CA-AMPK mice compared to wild type littermates on standard chow. This confirms the ability of improved endothelium-dependent relaxations in these transgenic mice under non-obese conditions as initially reported for the carotid artery with regenerated endothelium (Li *et al.*, 2012). By contrast, the parallel experiments with responses to acetylcholine in the presence of L-NAME did not reveal such effects of the CA-AMPK genotype, indicating a lack of impact on the endothelium-dependent hyperpolarization (EDH)-mediated relaxations known in this preparation (Gendron *et al.*, 2007). This is in contrast to findings with nitric oxide-independent amelioration of vascular function in lean aged mice by exogenous and not endothelium-specific activation of AMPK suggesting endothelium-dependent hyperpolarisation (Lesniewski *et al.*, 2012). Nitric oxide may counteract endothelium-dependent hyperpolarization (Najibi *et al.*, 1994; Olmos *et al.*, 1995; Bauersachs *et al.*, 1996). Hence, if the endothelial nitric oxide pathway is activated in CA-AMPK mice (Li *et al.*, 2012), endothelium-dependent responses may rely preferentially on it and alternative pathways such as EDH-mediated relaxations could be blunted.

The possible importance of NO presence in CA-AMPK mice is indicated by the degree of endothelium-dependent prostanoid-mediated contractions in responses to acetylcholine. In thoracic aortic rings of obese CA-AMPK mice, contractions induced by higher concentrations of acetylcholine were minimal as in their wild type littermates on continuous

high fat diet. This indicates the propensity of nitric oxide to counteract endothelium-dependent contractions (Auch-Schwelk *et al.*, 1992; Tang *et al.*, 2005a) possibly by preventing prostanoid formation (Bauersachs *et al.*, 1996). Furthermore, the NO system may need to be functional and stimulated to unmask the protective effects of an activated AMPK pathway in endothelial cells (Davis *et al.*, 2006; Li *et al.*, 2012). This is emphasized by the results in carotid arteries incubated with the nitric oxide synthase inhibitor L-NAME, where acetylcholine-induced endothelium-dependent contractions were exacerbated in quiescent preparations of obese CA-AMPK mice. By contrast, AMPK activation suppresses the production of vasoconstrictor prostanoids and thus improves vascular function in a rat model of type 2 diabetes (Matsumoto *et al.*, 2008), and there is a link between the cyclooxygenase inhibitors salicylate/aspirin and AMPK (Hawley *et al.*, 2012). However, the concentration of salicylate required to induce the AMPK pathway is much higher (Hawley *et al.*, 2012) than the amount of the structurally different indomethacin used in the present study to curtail prostanoid formation. In quiescent arteries of obese CA-AMPK mice (even if pooled for all samples from animals with the CA-AMPK genotype irrespective of the phenotype), the prostanoid levels, in particular those of the main representative prostacyclin (Rapoport & Williams, 1996; Gluais *et al.*, 2005; Baretella *et al.*, 2014; Baretella & Vanhoutte, 2016; Baretella *et al.*, 2017), were not significantly different from those obtained in wild type preparations, but cyclooxygenase inhibition by $10^{-6}$ mol/L indomethacin appears not to be complete in carotid arteries from obese CA-AMPK mice. Indeed, an induction of cyclooxygenase-2 has been observed in vascular tissues from these animals at a young age after a total of 16 weeks of high fat feeding (Liang *et al.*, 2014), but not of cyclooxygenase-1, which is responsible for EDCF-mediated responses in murine arteries (Tang *et al.*, 2005b). Further, the present findings suggest that the augmented prostanoid-mediated contractions in preparations from obese CA-AMPK can be attributed to increased TP receptor

responsiveness. In confirmation of this interpretation, unlike in their wild type littermates, the sensitivity to TP receptor activation – as evaluated with contractions to the full TP receptor agonist U46619 – was not decreased in carotid artery preparations of obese CA-AMPK mice. However, the observation of the incomplete inhibition of prostanoid release by $10^{-6}$ mol/L indomethacin upon stimulation with acetylcholine, does not exclude a possible contribution of endogenous prostanoids in the responses to U46619 in the preparations from these obese transgenic mice and a contribution of prostanoids other than prostacyclin, the main representative identified from rodent arteries (Rapoport & Williams, 1996; Gluais *et al.*, 2005), remains possible.

A major finding of the present study is the impact of change in diet on improved vascular function in terms of reduced $\alpha_1$-adrenergic contractions in main renal arteries in the absence and presence of L-NAME, and in mice of either genotype; this indicates that there is no alleviation by nitric oxide and continuous activation of the AMPK pathway in endothelial cells, respectively. Taken in conjunction, the present findings indicate that up to two years old mice with or without diet-induced obesity were normotensive, normoglycaemic, and had similar vascular function except for an augmented sensitivity to $\alpha_1$-adrenergic activation in main renal arteries of obese animals. This effect was absent in mice with the dietary regimen changed to standard chow following thirty weeks of high fat feeding, demonstrating its reversibility upon weight loss, and was not affected by endothelial overexpression of CA-AMPK.

As demonstrated, long-term obesity *per se* is not sufficient to cause endothelial dysfunction in mice, which may need additional cardiovascular risk factors such as diabetes, inflammation or dyslipidemia/atherosclerosis (Barton *et al.*, 1998; Li *et al.*, 2012; Liang *et al.*, 2013; Miller *et al.*, 2013). In conclusion, as in obese patients (Wycherley *et al.*, 2010), the

findings of the present study highlight the importance of weight loss by changes in diet to prevent vascular dysfunction in the long-term.

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## Figure Captions

**Fig. 1** Growth curves of CA-AMPK mice and their wild type (WT) littermates receiving either high fat diet to induce obesity or standard chow for lean control animals (*n*=13-20). Some of the mice on high fat diet were changed to standard chow (*n*=5), while others were kept on high fat diet (*n*=8). Data are given in absolute values and shown as means ± SEM, which are within the symbols if not visible.

**Fig. 2** Responses to the endothelium-dependent agonist acetylcholine in thoracic aortic rings of lean (*n*=4), *obese to lean* (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates (**a**) and to the nitric oxide donor sodium nitroprusside (**b**). Data are expressed as changes in tension relative to pre-contractions to phenylephrine (PE) and shown as means ± SEM

**Fig. 3** Relaxations to the endothelium-dependent agonist acetylcholine in segmental renal artery rings of lean (*n*=4), *obese to lean* (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates. Experiments were performed in the presence of indomethacin ($10^{-6}$ mol/L) only (**a**), indomethacin and L-NAME ($3x10^{-4}$ mol/L) (**b**), or indomethacin, L-NAME, and apamin *plus* charybdotoxin (both $10^{-7}$ mol/L) (**c**). Data are shown as changes in tension relative to pre-contractions to phenylephrine (PE) and expressed as means ± SEM. **P*<0.05 *versus* lean WT littermates; †*P*<0.05 $E_{max}$ *versus* obese to lean WT littermates

**Fig. 4** Prostanoid-mediated contractions to the endothelium-dependent agonist acetylcholine in carotid arterial rings of lean (*n*=4), *obese to lean* (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates in the presence of L-NAME ($3x10^{-4}$ mol/L) (**a**), or

in the presence of indomethacin ($10^{-6}$ mol/L) and L-NAME (**b**). Prostanoid (prostacyclin metabolite 6-keto prostaglandin $F_{1\alpha}$ [6-keto $PGF_{1\alpha}$], prostaglandin $E_2$ [$PGE_2$], and thromboxane $A_2$ metabolite thromboxane $B_2$ [$TXB_2$]) levels (**c**) of solution samples from these experiments without (empty bars) or with (full bars) indomethacin present were determined. Data are expressed in percentage of reference contractions to 60 mmol/L high potassium solution (**a** and **b**) or as picogram (pg) normalized for one milliliter (mL) myograph solution (**c**) and shown as means ± SEM. ***P*<0.05 *versus* obese WT littermates, *obese to lean* CA-AMPK, and lean controls, **†***P*<0.05 *versus* experiments without indomethacin in each individual group

**Fig. 5** Contractions to the full TP receptor agonist U46619 in carotid arterial rings of lean (*n*=4), *obese to lean* (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates. Experiments were performed in the presence of L-NAME ($3x10^{-4}$ mol/L) and indomethacin ($10^{-6}$ mol/L). Data are expressed in percentage of reference contractions to 60 mmol/L high potassium solution and shown as means ± SEM

**Fig. 6** Contractions to the $\alpha_1$-adrenergic agonist phenylephrine in main renal arteries of wild type (WT; *n*=4-6) and CA-AMPK (*n*=3-4) mice fed standard chow (lean), high fat diet before change to standard chow (*obese to lean*), or continuously on high fat diet (obese). Experiments were performed in the absence (**a**) or presence (**b**) of L-NAME ($3 \times 10^{-4}$ mol/L) Data are expressed in percentage of reference contractions to 60 mmol/L high potassium solution and shown as means ± SEM. ***P*<0.01 obese *versus* lean, **†***P*<0.05 obese *versus* obese to lean

**Supplemental Fig. 1** Typical genotyping gel image (1.5% Agarose): Samples from mice with endothelial over-expression of adenosine monophosphate-activated protein kinase (CA-AMPK) show a strong band at 250 base pairs (bp), whereas wild type (WT) littermates have unspecific band(s) with higher size, that do not appear in lysates from transgenic animals.

**Supplemental Fig. 2** Survival of lean CA-AMPK and WT control mice fed standard chow (*n*=9-11).

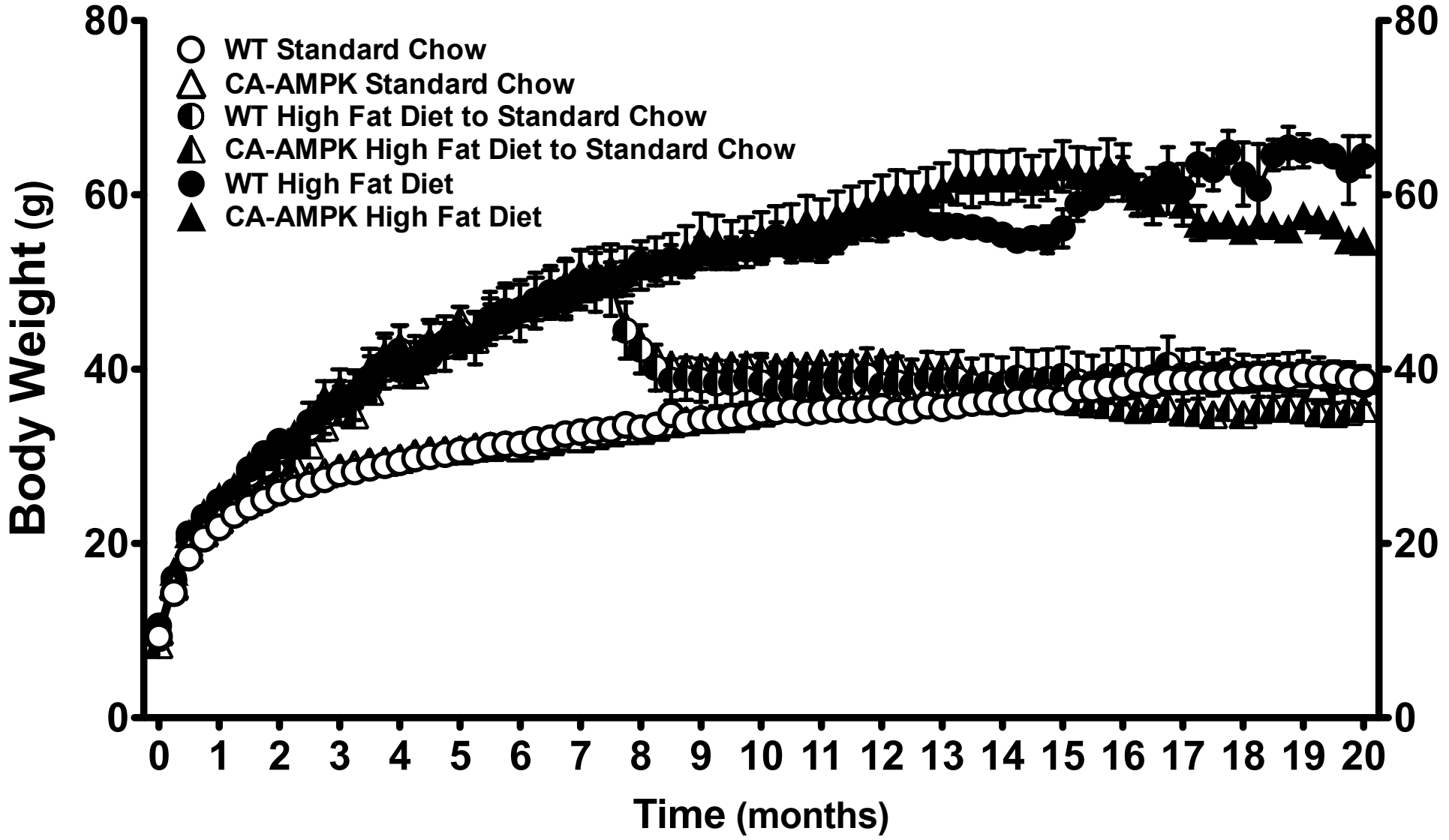

WT Standard Chow
CA-AMPK Standard Chow
WT High Fat Diet to Standard Chow
CA-AMPK High Fat Diet to Standard Chow
WT High Fat Diet
CA-AMPK High Fat Diet
Body Weight (g)
0
20
40
60
80
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time (months)

Fig. 1

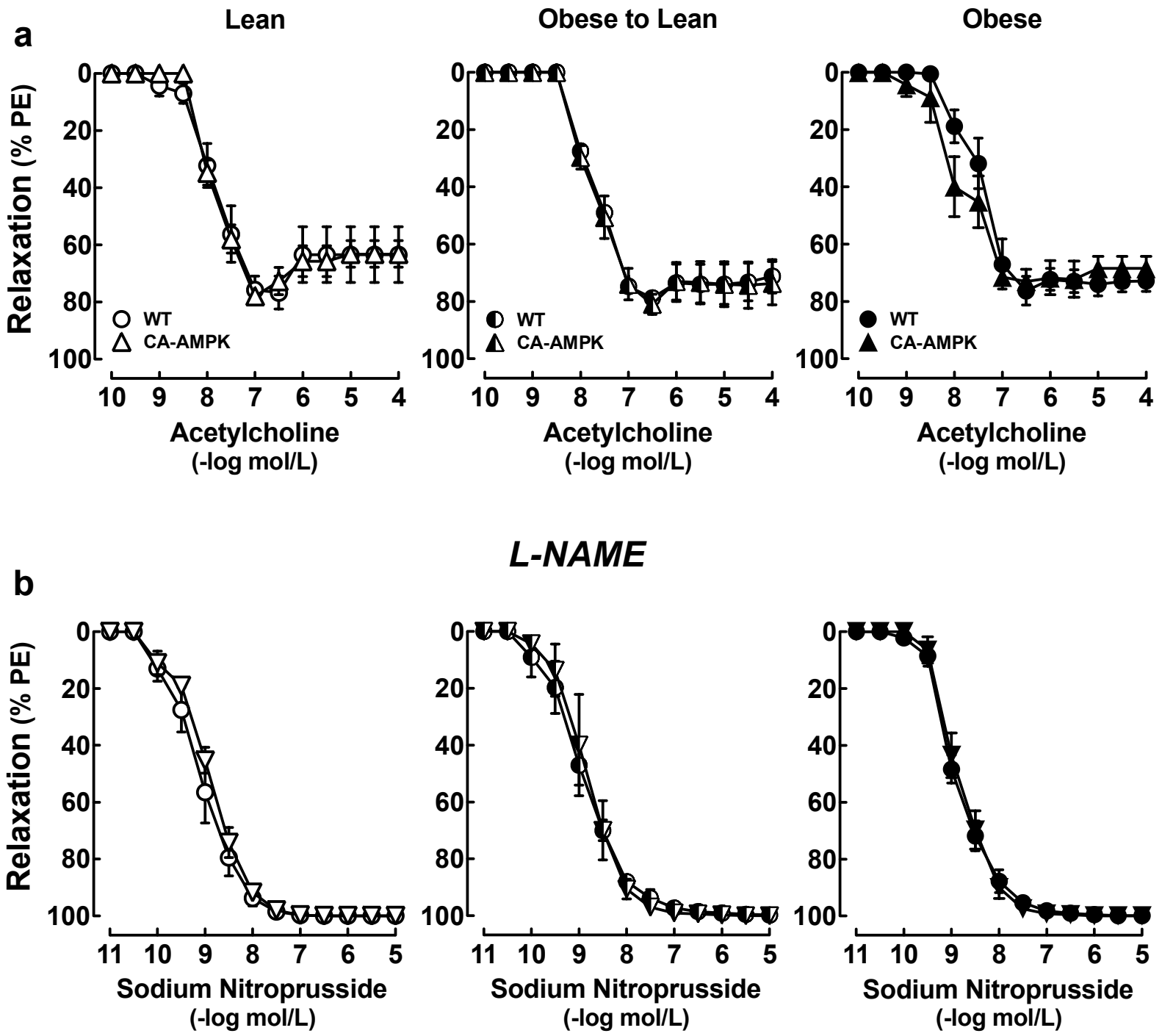

a
Lean
Obese to Lean
Obese
Relaxation (% PE)
0
20
40
60
80
100
WT
CA-AMPK
10 9 8 7 6 5 4
Acetylcholine
(-log mol/L)
L-NAME
b
11 10 9 8 7 6 5
Sodium Nitroprusside
(-log mol/L)

Fig. 2

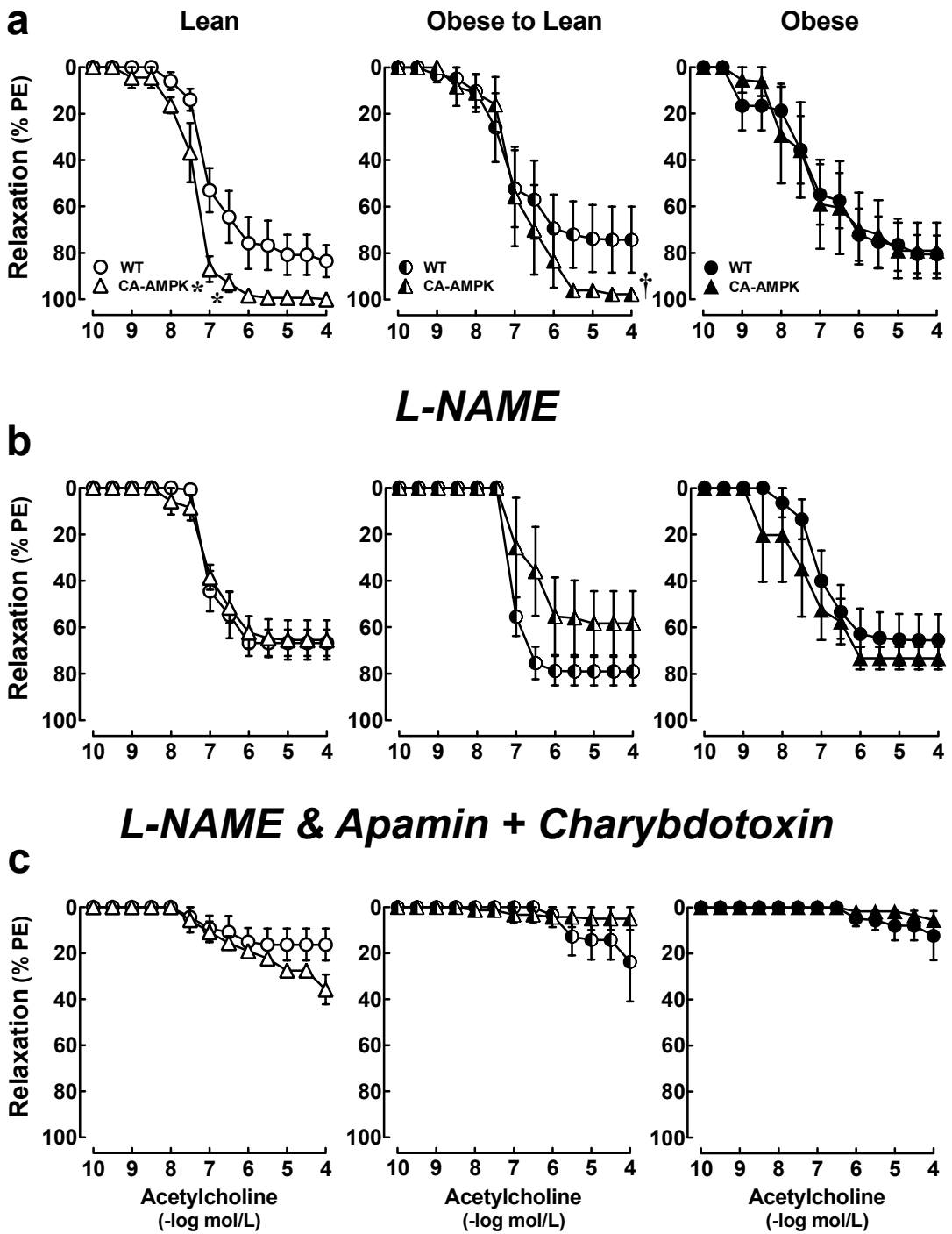

a
Lean
Obese to Lean
Obese
Relaxation (% PE)
WT
CA-AMPK
L-NAME
b
L-NAME & Apamin + Charybdotoxin
c
Acetylcholine
(-log mol/L)

Fig. 3

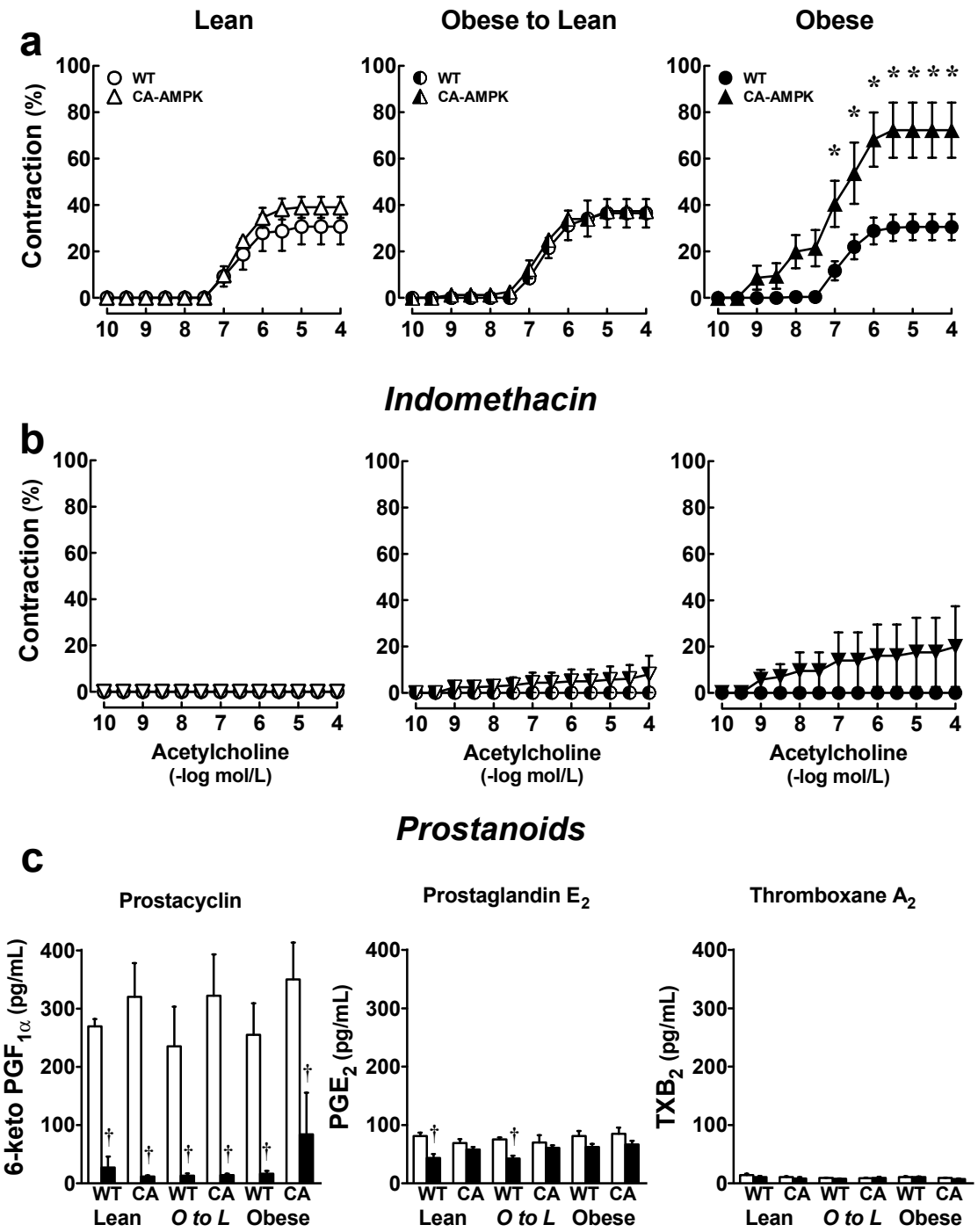

a
Lean
Obese to Lean
Obese
WT
CA-AMPK
Contraction (%)
Indomethacin
b
Acetylcholine
(-log mol/L)
Prostanoids
c
Prostacyclin
Prostaglandin E2
Thromboxane A2
6-keto PGF1α (pg/mL)
PGE2 (pg/mL)
TXB2 (pg/mL)
WT CA WT CA WT CA
Lean O to L Obese

Fig. 4

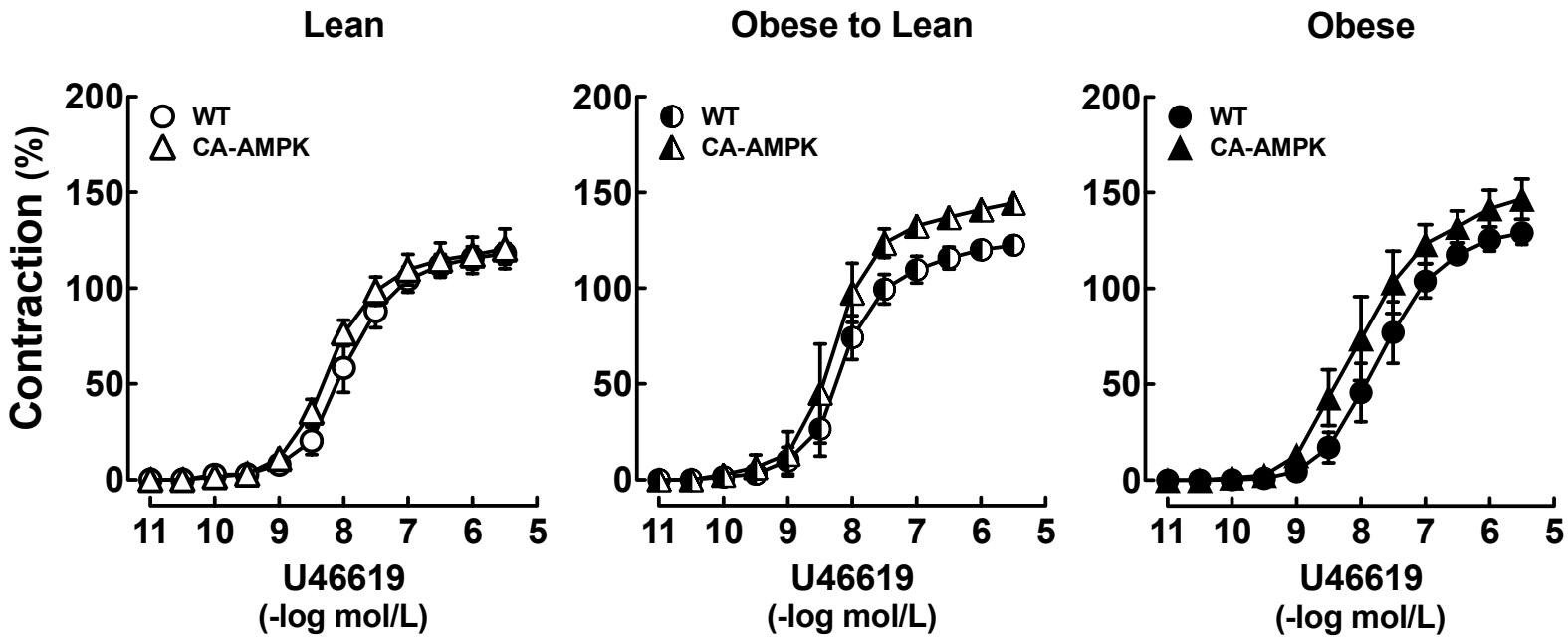

Lean
Obese to Lean
Obese
Contraction (%)
200
150
100
50
0
WT
CA-AMPK
11 10 9 8 7 6 5
U46619
(-log mol/L)

Fig. 5

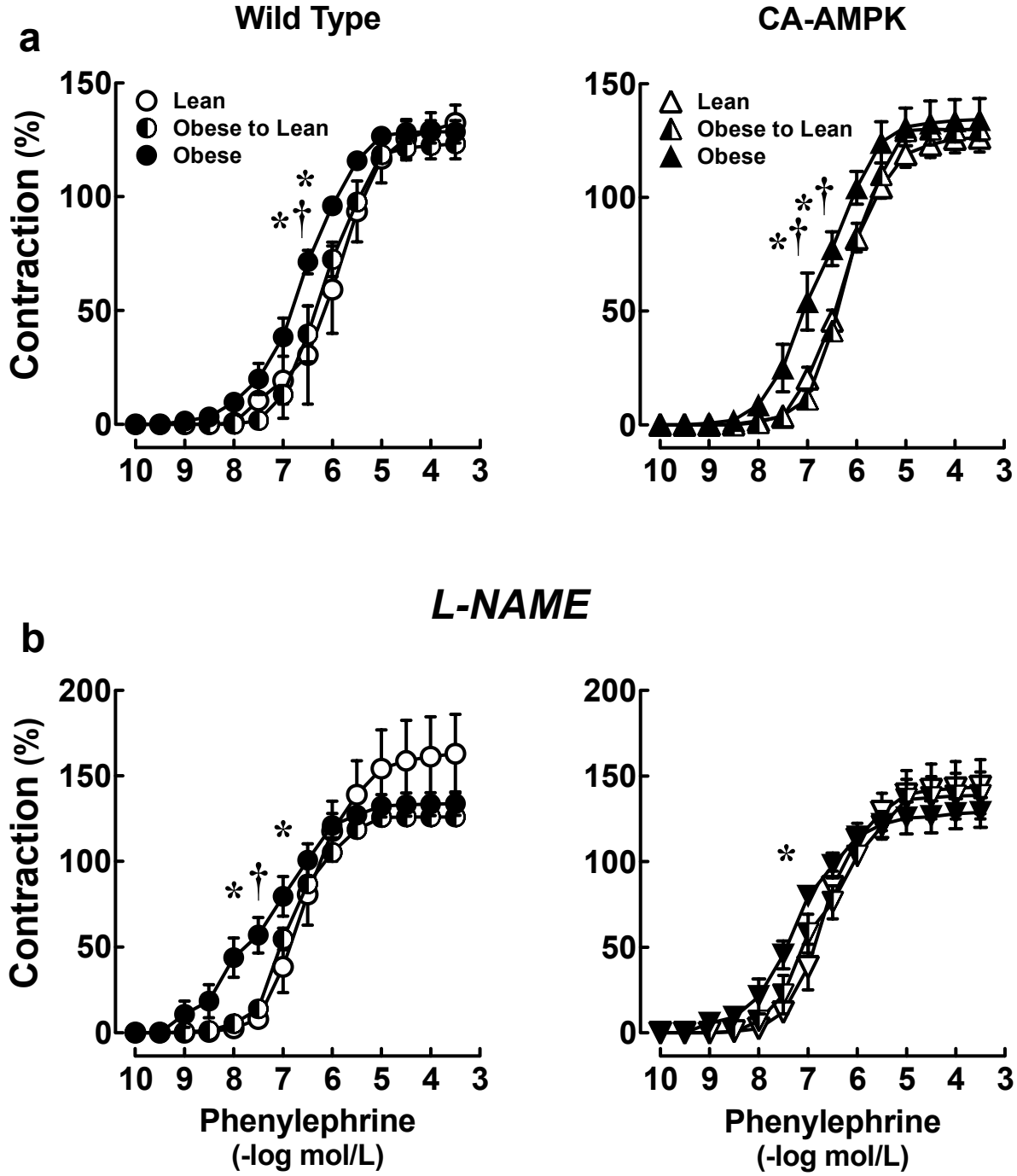

a
Wild Type
CA-AMPK
Lean
Obese to Lean
Obese
Contraction (%)
150
100
50
0
10 9 8 7 6 5 4 3
*
*†
L-NAME
b
200
150
100
50
0
Phenylephrine
(-log mol/L)

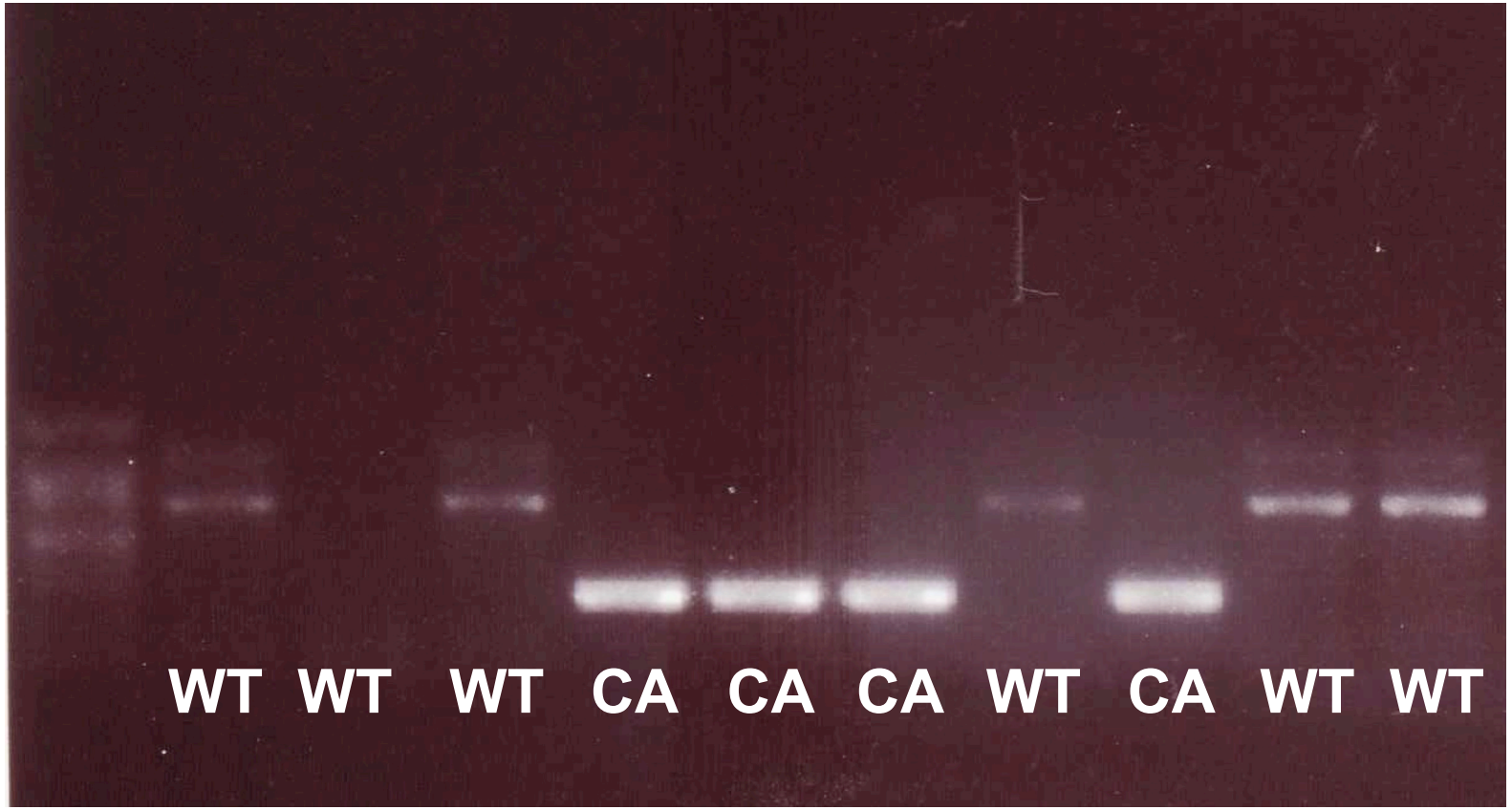
Fig. 6

**Table 1** Cardiometabolic parameters in *lean* (*n*=4), previously obese changed to standard chow (*obese to lean*; *n*=3-4), and continuously high fat diet fed *obese* CA-AMPK and wild type (WT) mice (*n*=4-6). Values are means ± SEM and in each parameter averaged for the three dietary treatment groups. *****$P<0.05$ obese *versus* lean.

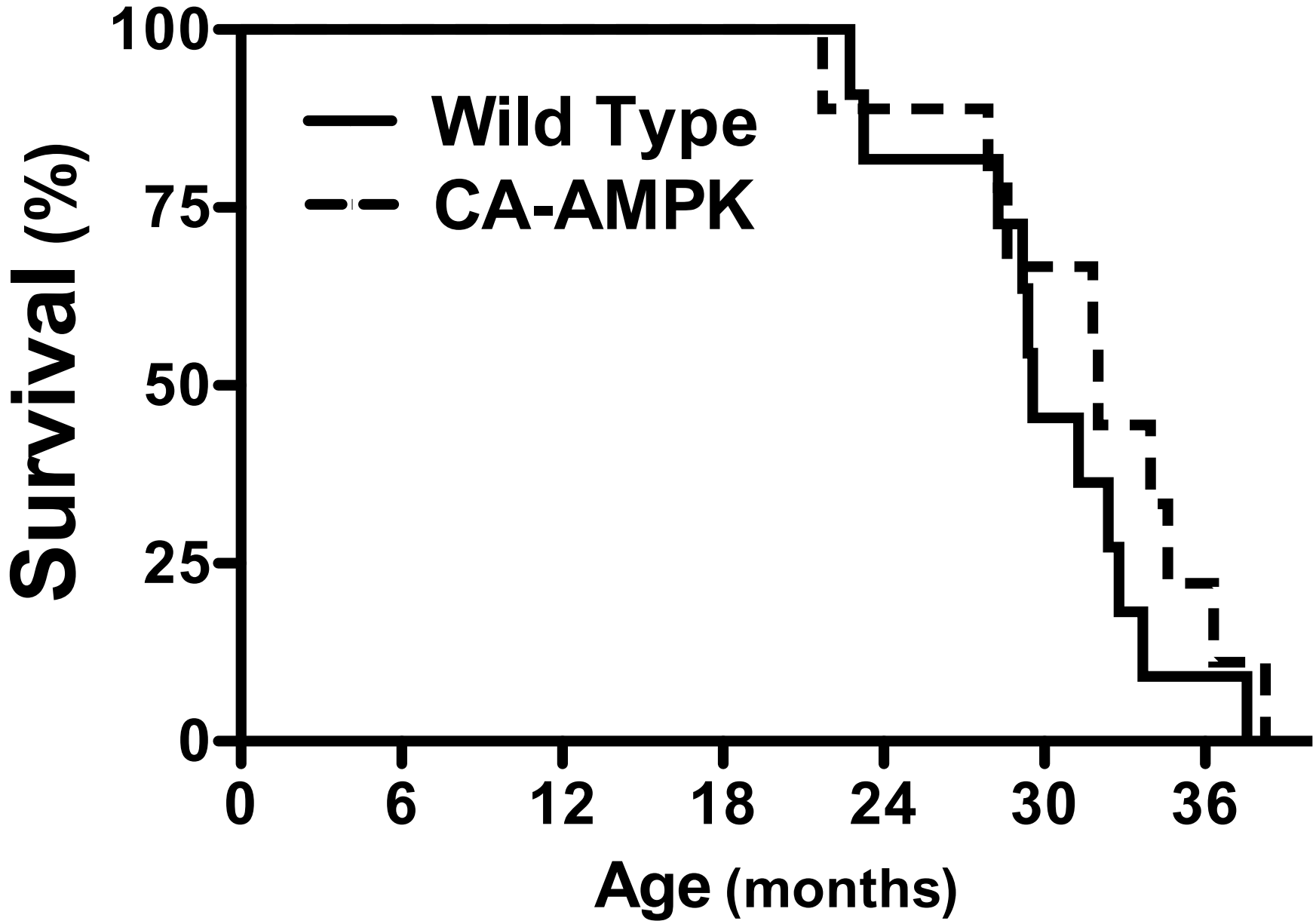
| Parameter | WT | AMPK | **LEAN** | WT | AMPK | **OBESE TO LEAN** | WT | AMPK | **OBESE** |
|---|---|---|---|---|---|---|---|---|---|
| Body weight (g) | 30±2 | 34±2 | 32±2 | 29±2 | 27±3 | 28±2 | 45±3 | 45±1 | 45±2* |
| Total cholesterol (mg/dL) | 61±11 | 47±2 | 54±6 | 50±8 | 61±5 | 54±5 | 105±21 | 100±15 | 103±13* |
| Triglycerides (mg/dL) | 45±8 | 59±5 | 52±5 | 48±4 | 40±9 | 44±4 | 57±5 | 53±8 | 55±4 |
| Adiponectin (µg/mL) | 24±2 | 21±2 | 23±2 | 24±5 | 21±4 | 23±3 | 20±3 | 18±3 | 19±2 |
| Insulin (ng/mL) | 1.0±0.3 | 1.3±0.3 | 1.2±0.2 | 1.9±0.6 | 0.2±0.1 | 1.3±0.5 | 3.7±1.0 | 3.0±1.0 | 3.4±0.7* |
| Glucose (mmol/L) | 4.8±0.9 | 5.2±0.6 | 5.0±0.5 | 4.5±1.1 | 4.3±0.8 | 4.4±0.6 | 6.4±0.7 | 5.4±0.3 | 6.0±0.5 |
| Systolic blood pressure (mmHg) | 105±5 | 104±4 | 104±3 | 117±2 | 111±5 | 114±2 | 106±2 | 108±7 | 106±3 |
| Heart rate (beats/min) | 634±18 | 660±15 | 649±12 | 651±30 | 669±35 | 658±20 | 681±6 | 709±18 | 684±5* |
| Heart weight (mg) | 174±4 | 180±5 | 177±3 | 149±9 | 179±3 | 162±8 | 218±17 | 207±4 | 214±10* |
| Heart (%Body weight) | 0.59±0.03 | 0.54±0.04 | 0.56±0.02 | 0.52±0.02 | 0.67±0.09 | 0.58±0.05 | 0.50±0.04 | 0.45±0.04 | 0.48±0.03 |

**Table 2** $pD_2$ values of contractions to the full TP receptor agonist U46619 in thoracic aortic-, carotid-, and main renal arterial rings of lean (*n*=4), obese to lean (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates. Experiments were performed in the presence of L-NAME ($3x10^{-4}$ mol/L) and indomethacin ($10^{-6}$ mol/L). Data are expressed as negative logarithms of the calculated $EC_{50}$ values and shown as means ± SEM. **P*<0.05 *versus* lean WT littermates; †*P*<0.05 *versus* obese WT littermates; ‡*P*<0.01 *versus* obese to lean WT, and *P*<0.05 *versus* lean WT controls.

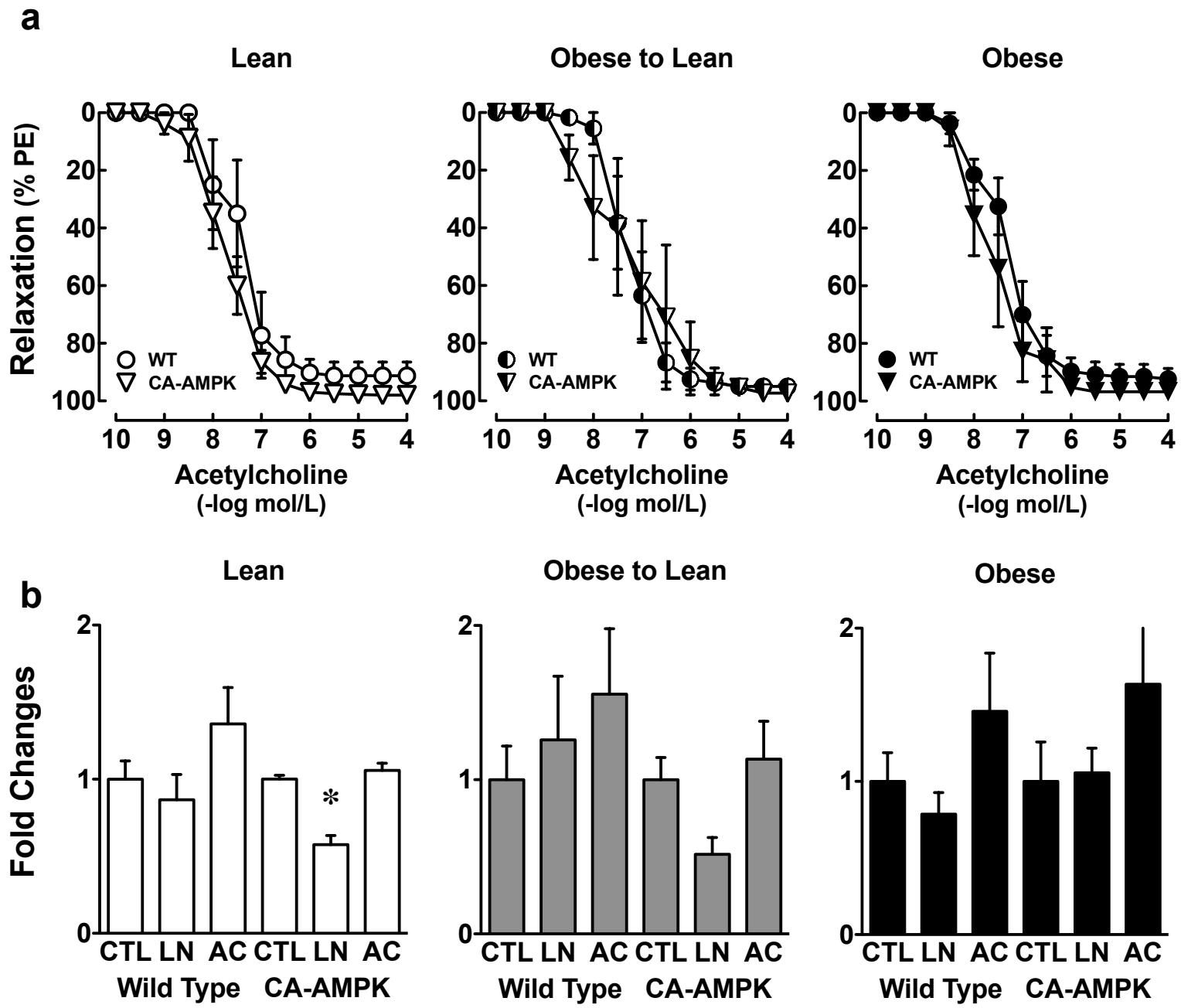
| Preparation | WT | AMPK | WT | AMPK | WT | AMPK |
|---|---|---|---|---|---|---|
| | LEAN | | OBESE TO LEAN | | OBESE | |
| Thoracic aorta | 8.5±0.1 | 8.4±0.1 | 8.6±0.1 | 8.4±0.0 | 8.4±0.1 | 8.5±0.1 |
| Carotid artery | 8.0±0.1 | 8.2±0.1* | 8.1±0.1 | 8.3±0.1 | 7.7±0.1‡ | 8.0±0.1† |
| Main renal artery | 8.6±0.3 | 8.4±0.1 | 8.6±0.1 | 8.4±0.2 | 8.4±0.1 | 8.6±0.3 |




**Supplemental Fig. 1**




Supplemental Fig. 2




Supplemental Fig. 3

**Supplemental Table 1** Reference contractions in response to 60 mmol/L high potassium ($K^+$) depolarizing solution in isolated rings of thoracic aortae, carotid arteries, main- and segmental renal arteries from lean, obese to lean, and obese CA-AMPK mice and wild type (WT) littermates (*n*=3-6). Data are expressed in milliNewtons (mN) and shown as means ± SEM. *****$P$<0.05 obese *versus* obese to lean, †$P$<0.05 obese to lean *versus* lean.

| Artery | WT | AMPK | *WT* | *AMPK* | WT | AMPK |
|---|---|---|---|---|---|---|
| | Lean | | *Obese to Lean* | | Obese | |
| **Thoracic Aorta** | 15±1 | 16±1 | *17±1* | *20±2* | 11±1* | 11±1* |
| **Carotid** | 6±1 | 6±1 | *5±1* | *6±1* | 6±1 | 6±1 |
| **Main Renal** | 5±1 | 6±1 | *8±1†* | *7±1* | 7±1 | 8±1 |
| **Segmental Renal** | 5±1 | 5±1 | *5±1* | *5±1* | 5±1 | 6±1 |

**Supplemental Table 2** $pD_2$ values of contractions to the $\alpha_1$-adrenoceptor agonist phenylephrine in thoracic aortic- and main renal arterial rings of lean (*n*=4), obese to lean (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates. Experiments were performed in the absence or presence of L-NAME ($3x10^{-4}$ mol/L). Data are expressed as negative logarithms of the calculated $EC_{50}$ values and shown as means ± SEM. **P*<0.05 *versus* lean WT littermates; †*P*<0.001 *versus* lean WT littermates; ‡*P*<0.05 *versus* lean AMPK controls.

| Preparation | WT | AMPK | WT | AMPK | WT | AMPK |
|---|---|---|---|---|---|---|
| | LEAN | | OBESE TO LEAN | | OBESE | |
| Thoracic aorta | 6.3±0.1 | 6.4±0.1 | 6.6±0.1* | 6.7±0.2 | 6.6±0.2 | 6.7±0.1 |
| + L-NAME | 6.6±0.1 | 6.7±0.1 | 6.9±0.1* | 6.8±0.1 | 6.7±0.1 | 6.7±0.1 |
| Main renal artery | 5.9±0.1 | 6.3±0.0* | 6.1±0.1 | 6.2±0.0 | 6.6±0.1† | 6.7±0.1‡ |
| + L-NAME | 6.5±0.1 | 6.6±0.1 | 6.8±0.0* | 6.7±0.1 | 7.4±0.1* | 7.2±0.1‡ |

**Supplemental Table 3** $pD_2$ values of relaxations to acetylcholine in phenylephrine-contracted segmental renal arterial rings (with endothelium) of lean (*n*=4), obese to lean (*n*=3-4), and obese (*n*=4-6) CA-AMPK mice and their wild type (WT) littermates. All experiments were performed in the absence of indomethacin ($10^{-6}$ mol/L), and some additionally with L-NAME ($3x10^{-4}$ mol/L) or apamin *plus* charybdotoxin (both $10^{-7}$ mol/L). Data are expressed as negative logarithms of the calculated $EC_{50}$ values and shown as means ± SEM. **P*<0.01 *versus* lean WT littermates; †*P*<0.05 *versus* obese to lean WT littermates and lean CA-AMPK controls; ‡*P*<0.05 *versus* obese WT littermates.

| Treatment | WT | AMPK | WT | AMPK | WT | AMPK |
|---|---|---|---|---|---|---|
| | LEAN | | OBESE TO LEAN | | OBESE | |
| Indomethacin | 7.1±0.1 | 7.4±0.0* | 7.3±0.2 | 7.0±0.1† | 7.4±0.3 | 7.6±0.4 |
| + L-NAME | 7.1±0.1 | 7.0±0.1 | 7.1±0.1 | 6.8±0.2 | 7.1±0.2 | 7.5±0.3 |
| + Apamin *plus* Charybdotoxin | 7.5±0.1 | 7.7±0.1 | 7.3±0.1 | 7.4±0.3 | 7.4±0.1 | 7.7±0.1‡ |