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Reduced L-Carnitine Transport in Aortic Endothelial Cells from Spontaneously Hypertensive Rats

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Abstract

Impaired L-carnitine uptake correlates with higher blood pressure in adult men, and L-carnitine restores endothelial function in aortic rings from spontaneously hypertensive rat (SHR). Thus, endothelial dysfunction in hypertension could result from lower L-carnitine transport in this cell type. L-Carnitine transport is mainly mediated by novel organic cation transporters 1 (Octn1, Na^+-independent) and 2 (Octn2, Na^+-dependent); however, their kinetic properties and potential consequences in hypertension are unknown. We hypothesize that L-carnitine transport kinetic properties will be altered in aortic endothelium from spontaneously hypertensive rats (SHR). L-Carnitine transport was measured at different extracellular pH (pH_o 5.5–8.5) in the absence or presence of sodium in rat aortic endothelial cells (RAECs) from non-hypertensive Wistar-Kyoto (WKY) rats and SHR. Octn1 and Octn2 mRNA relative expression was also determined. Dilation of endothelium-intact or denuded aortic rings in response to calcitonine gene related peptide (CGRP, 0.1–100 nmol/L) was measured (myography) in the absence or presence of L-carnitine. Total L-carnitine transport was lower in cells from SHR compared with WKY rats, an effect due to reduced Na^+-dependent (Na^+_{dep}) compared with Na^+-independent (Na^+_{indep}) transport components. Saturable L-carnitine transport kinetics show maximal velocity (V_{max}), without changes in apparent K_m for Na^+_{indep} transport in SHR compared with WKY rats. Total and Na^+_{dep} component of transport were increased, but Na^+_{indep} transport was reduced by extracellular alkalization in WKY rats. However, alkalization reduced total and Na^+_{indep} transport in cells from SHR. Octn2 mRNA was higher than Octn-1 mRNA expression in cells from both conditions. Dilation of artery rings in response to CGRP was reduced in vessels from SHR compared with WKY rats. CGRP effect was endothelium-dependent and restored by L-carnitine. All together these results suggest that reduced L-carnitine transport (likely via Na^+-dependent Octn2) could limit this compound's potential beneficial effects in RAECs from SHR.

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Introduction

Essential hypertension is characterized by high blood pressure without an identifiable primary cause [1,2]. Oral administration of L-carnitine, a natural amino acidic compound, in patients with hypertension resulted in an improvement of arterial blood pressure, thus suggesting a beneficial vascular role for this compound in these subjects [3,4]. Along with the well-described role of L-carnitine on fatty acid mitochondrial metabolism [5,6], L-carnitine also increases the metabolic activity in human vascular endothelium [7,8], and improves the bioavailability of nitric oxide (NO) in rat aorta [9] and in fetal lamb pulmonary vasculature [10]. Other studies show that L-carnitine supplementation in healthy subjects improved postprandial flow-mediated dilation after a high-fat meal [11]. Taken together, all these findings suggest that the transport of L-carnitine into the endothelial cells could be an essential, limiting step of its potential beneficial biological effects in hypertension.

The uptake of L-carnitine is mediated by novel organic cation transporters (OCTNs) of which at least three isotypes (rOctn1, rOctn2 and rOctn3) are expressed in rats [12]. Octn1- an Octn3-mediated L-carnitine transport is independent of sodium (Na^+) with apparent Michaelis-Menten (K_m) values in the range of 2–200 μmol/L [13–15] and 3–6 μmol/L [16,17], respectively. On the contrary, Octn2-mediated transport is Na^+-dependent with

apparent K_m between 2–20 μmol/L [16,18–23]. Studies performed in spontaneously hypertensive rat (SHR) show that L-carnitine restores endothelial function in preparations of aortic rings [24,25], and ameliorates the high-systolic arterial blood pressure exhibited by hypertensive animals [5,8,26]. Interestingly, there are no reports addressing the properties of L-carnitine transport in endothelial cells from SHR. Thus, we hypothesize that the activity of OCTNs-mediated membrane transport of L-carnitine by the aortic endothelium is reduced in these hypertensive animals.

The results of this study show that aorta endothelial cells from SHR exhibit reduced maximal L-carnitine transport capacity compared with cells from non-hypertensive animals. Transport of L-carnitine was saturable and mediated by a larger Na^+-independent, Octn1-like compared with a Na^+-dependent, Octn2-like transport activity in these cells from SHR. However, similar Na^+-dependent and Na^+-independent transport components were found in non-hypertensive rats. It is suggested that the observed endothelial dysfunction in SHR could be due to reduced Na^+-dependent transport of L-carnitine, which could limit the potential beneficial effects of this compound in the endothelial function in hypertension.

Methods

Ethics statement and animals

This investigation strictly conforms to the principles outlined in the European Union Guidelines on the protection of animals used for scientific purposes (DIRECTIVE 2010/63/EU of the European Parliament and of the Council). Protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Sevilla (Spain). Normotensive male Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) aged 8 weeks were obtained from the French Animal Production Center, JANVIER S.A.S. (Saint Berthevin Cedex, France). Rats were housed at a temperature of 22–24°C in individual cages and freely fed (*ad libitum*) regular pellet diet (12 mm pellet, Harlan Laboratories, Indianapolis, USA) until they were 10 weeks of age (wa). They were divided into two groups of 15 animals each, i.e., WKY (control) and SHR (hypertensive) animals. Characterization of WKY rats and SHR in terms of the diastolic and systolic blood pressures and body weight was determined at arrival of the animals (8 wa) and at the moment of isolation of aorta endothelial cells (i.e., 10 wa) as reported [26]. Diastolic and systolic blood pressure values were not significantly different ($P>0.05$) at 8 wa (diastolic = 93 ± 3 mmHg, systolic = 123 ± 6 mmHg) compared with 10 wa (diastolic = 95 ± 2 mmHg, systolic = 124 ± 5 mmHg) in WKY rats. However, these parameters were elevated ($P<0.01$) in SHR at 8 wa (diastolic = 190 ± 2 mmHg, systolic = 233 ± 2 mmHg) and 10 wa (diastolic = 191 ± 2 mmHg, systolic = 231 ± 1 mmHg) compared with the corresponding systolic or diastolic values in WKY rats, but were not significantly different ($P>0.05$) between them in SHR.

Cell culture

Aortas from WKY rats and SHR aged 10 weeks were excised and placed in a petri dish containing phosphate-buffered saline (PBS) solution ((mmol/L) NaCl 130, KCl 2.7, Na_2HPO_4 0.8, KH_2PO_4 1.4 (pH 7.4, 4°C)). The tissue was rinsed by changing PBS until free of any visible blood, and the aorta was stripped of adventicia as reported [27]. Rat aorta endothelial cells (RAECs) were isolated by scraping of rat aortic lumen in the presence of medium 199 (M199) (Gibco Life Technologies, Carlsbad, CA, USA) containing 5 mmol/L D-glucose, 10% new born calf serum, 10% fetal calf serum (FCS) (Gibco), 3.2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin (primary culture medium, PCM) (Gibco), and cultured up to passage 3 (37°C, 5% CO_2). Twenty-four hours prior to experiments the incubation medium was replaced by M199 containing 2% sera after two rinses with 200 μL in PBS (37°C). Cells were used in passage 3 for most of the experiments. In addition, in some of transport assays confluent freshly isolated cells (i.e., passage 0) or cells in passages 1 or 2 in culture were also used.

L-Carnitine transport

Total transport of L-carnitine (*TTC*) was defined as the result of the sum of the Na^+-dependent (hereafter referred as ${}^{TTC}Na^+{}_{dep}$) and Na^+-independent (hereafter referred as ${}^{TTC}Na^+{}_{indep}$) components plus a nonsaturable, lineal component of transport in the range of L-carnitine used in this study (hereafter referred as $m\bullet[Car]$, where m corresponds to slopes of lineal phases of *TTC* at each L-carnitine concentrations [*Car*]) [28]. The *TTC* (0–80 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine (NEN, Dreieich, FRG), 30 seconds, 37°C) was measured as previously described for other amino acids in primary cultured endothelium [28]. Briefly, *TTC* assays were performed in a Na^+-containing Krebs ((mmol/L): NaCl 131, KCl 5.6, $NaHCO_3$ 25, NaH_2PO_4 1, Hepes 20, D-glucose 5, $CaCl_2$ 2.5, $MgCl_2$ 1 (pH 7.4, 37°C)) or in Na^+-free Krebs solution ((mmol/L) *N*-methyl-D-glucamine (NMDG) 120, KCl 5.6, Hepes 20, D-glucose 5, $CaCl_2$ 2.5, $MgCl_2$ 1 (pH 7.4, 37°C)).

Initial rate for *TTC*, ${}^{TTC}Na^+{}_{dep}$ and ${}^{TTC}Na^+{}_{indep}$ components was derived from slopes of lineal phases of 20 μmol/L L-carnitine transport. Values for L-carnitine transport were adjusted to the one phase exponential association equation considering the least squares fit:

$$v_i = V_m\bullet\left(1-e^{-(k\bullet t)}\right)$$

where v_i is initial velocity, V_m is mayor velocity at a given time (t) and L-carnitine concentration, and e and k are constants.

Data for *TTC*, ${}^{TTC}Na^+{}_{dep}$ and ${}^{TTC}Na^+{}_{indep}$ components at initial rates (i.e., linear uptake up to 30 seconds) was adjusted to the Michaelis-Menten hyperbola plus a nonsaturable, lineal component ($m\bullet[Car]$) as described [28]:

$$\frac{v}{Vmax} = \frac{[Car]}{Km+[Car]} + (m\bullet[Car])$$

where v is the initial reaction velocity relative to the maximal velocity (V_{max}) and apparent Michaelis-Menten parameter (K_m) of transport at a given L-carnitine concentration ([*Car*]), m represents the slope of transport for the range of 0–80 μmol/L L-carnitine and $m\bullet[Car]$ is the nonsaturable, lineal component of transport in the range of L-carnitine used in this study [28]. Each assay was run in duplicate with transport activity expressed as pmol/μg protein/minute.

After subtracting the $m\bullet[Car]$ component from *TTC* the remaining transport was defined as total overall saturable transport of L-carnitine (*TSC*). Transport in Na^+-free Krebs was considered as the Na^+-independent component of *TSC* (${}^{TSC}Na^+{}_{indep}$) and the Na^+-component (${}^{TSC}Na^+{}_{dep}$) was derived from:

$${}^{TSC}Na^+_{dep} = TSC - {}^{TSC}Na^+_{indep}$$

The kinetic parameters V_{max} and apparent K_m for the *TSC*, $^{TSC}Na^+_{dep}$ or $^{TSC}Na^+_{indep}$ components were estimating by fitting the data to the single Michaelis-Menten asymptotic hyperbola equation:

$$\frac{v}{Vmax} = \frac{[Car]}{Km + [Car]}$$

Cells were exposed to PCM 2% sera for a period of 2 hours before the transport assays were performed. Cell viability was assayed by Trypan blue exclusion and was not significantly altered (~96% of viable cells) in any experimental condition in this study. Rinsing the monolayers with ice-cold Krebs with or without Na^+ terminated the tracer uptake. Radioactivity in formic acid cell digests was determined by liquid scintillation counting in an automated low activity liquid scintillation analyzer (Tri-Carb 2810TR, PerkinElmer, Santa Clara, CA, USA) with efficiency estimated by converting counts to disintegrations per minute (d.p.m.) [28]. Uptake of L-[^{3}H]carnitine was corrected for its extracellular trapping by measuring the accumulation of the non-transportable D-[^{14}C]mannitol (1 μCi/mL) (PerkinElmer) in the extracellular space by:

$$^3H_{in} = {}^3H_{sample} - \frac{^{14}C_{sample} \bullet {}^{14}C_{st}}{^3H_{st}}$$

where $^3H_{in}$ is the L-[^{3}H]carnitine associated to the whole cell extracts, $^3H_{sample}$ and $^{14}C_{sample}$ are total L-[^{3}H]carnitine and D-[^{14}C]mannitol, respectively, for each sample analysed in the scintillation counter, and $^3H_{st}$ and $^{14}C_{st}$ are d.p.m. for standards of L-[^{3}H]carnitine and D-[^{14}C]mannitol, respectively.

The relative contribution of the hypertension exhibited by SHR to the saturable L-carnitine kinetic parameters ($1/F$) was estimated from the maximal transport capacity (V_{max}/K_m) values for *TSC* by:

$$\frac{1}{^{WKY/SHR}F} = \frac{^{WKY}K_m \bullet {}^{SHR}V_{max}}{^{WKY}V_{max} \bullet {}^{SHR}K_m}$$

where $^{WKY}V_{max}$ and $^{WKY}K_m$ are the kinetics parameters for *TSC* in cells from WKY rats, and $^{SHR}V_{max}$ and $^{SHR}K_m$ are kinetics parameters of transport in cells from SHR. The relative contribution of the $^{TSC}Na^+_{dep}$ or $^{TSC}Na^+_{indep}$ components to *TSC* in SHR or WKY rats was estimated from:

$$\frac{1}{^{TSC/X}F} = \frac{^{TSC}K_m \bullet {}^{X}V_{max}}{^{TSC}V_m max \bullet {}^{X}K_m}$$

where X represents the $^{TSC}Na^+_{dep}$ or $^{TSC}Na^+_{indep}$ components for the kinetics parameters V_{max} and K_m of transport compared with *TSC* values. The relative contribution of the $^{TSC}Na^+_{dep}$ transport compared with the $^{TSC}Na^+_{indep}$ component to L-carnitine transport in SHR or WKY rats was estimated from:

$$\frac{1}{^{Na^+_{dep}/Na^+_{indep}}F} = \frac{^{Na^+_{dep}}K_m \bullet {}^{Na^+_{indep}}V_{max}}{^{Na^+_{dep}}V_{max} \bullet {}^{Na^+_{indep}}K_m}$$

Extracellular pH dependency

To assay the effect of extracellular pH (pH_o) on *TSC*, $^{TSC}Na^+_{dep}$ or $^{TSC}Na^+_{indep}$ components of L-carnitine transport (20 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) the cells were incubated in Na^+-containing or Na^+-free Krebs adjusted to a final pH_o of 5.5, 6.5, 7.5 or 8.5 as described [20]. The pH_o in the Na^+-containing Krebs solution was adjusted with 1 N HCl or 1 N NaOH, while the Na^+-free Krebs solution was adjusted with 1 N HCl or 1 N KOH. The pH_o values were monitored with a pHmeter (Oakton Instrument, Vernon Hills, IL, USA) and tracer uptake was terminated as above.

Isolation of total RNA and reverse transcription

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality and integrity were insured by gel visualization and spectrophotometric analysis ($OD_{260/280}$), and RNA concentration was determined at 260 nm. Aliquots (1 μg) of total RNA were reversed transcribed into cDNA as described [28].

RT-PCR

Experiments were performed using a Light Cycler 480 Detection System (Roche Diagnostic, Barcelona, Spain) in a reaction mix containing 0.5 μmol/L primers and master mix provided in the brilliant SYBR green qPCR Master Mix (Stratagene, La Jolla, CA, USA). SecureStart *Taq* DNA polymerase was activated (15 minutes, 95°C), and assays included a 95°C denaturation (15 seconds), annealing (20 seconds) at 54°C, and extension (10 seconds) at 72°C (rOctn1, rOctn2 and GAPDH). Product melting temperature values were 86°C (rOctn1), 86°C (rOctn2) and 85°C (GAPDH). Oligonucleotide primers: rOctn1 (sense) 5′-TGATAGCCTTCCTGGGCGATTGG-3′, rOctn1 (*anti*-sense) 5′-AAGGAGCCACAGAGAACGCCTAC-3′, rOctn2 (sense) 5′-AGGAGCCCATCAGCACACCCACG-3′, rOctn2 (*anti*-sense) 5′-GACGAAGGACGGACGACAGGTGC-3′, GAPDH (sense) 5′-GCCAAAAGGGTCATCATCTCCGC-3′, GAPDH (*anti*-sense) 5′-GGATGACCTTGCCCACAGCCTTG-3′.

The relative mRNA level in each group was estimated from the $2^{-\Delta\Delta CT}$ method [29]. Data were analyzed using the Light Cycler 480 SW 1.5 relative quantification (delta-delta-Ct) study software (Roche Diagnostic, Barcelona, Spain) and gene expression levels were normalized to GAPDH and given as relative fold change [30]. The GAPDH mRNA level was not significantly altered ($P>0.05$, n = 15) in all experimental conditions used in this study (not shown).

Rat aorta reactivity

Ring segments of 2–4 mm in length were dissected from rat aorta in cold (4°C) PBS solution. Vessel rings were mounted in a myograph (610M Multiwire Myograph System, Danish Myo Technology A/S, Denmark) for isometric force measurements in a Krebs physiological solution ((mmol/L): NaCl 118.5, KCl 4.7, $NaHCO_3$ 25, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, D-glucose 5.5, 300 μmol/L L-arginine, pH 7.4). Artery rings were maintained at 37°C and constantly bubbled with a mixture of 95% O_2/5% CO_2. The optimal diameter for each vessel was adjusted through the determination of the maximal active response evoked by 62.5 mmol/L KCl [28]. Isometric force was measured in response to calcitonine gene related peptide (CGRP, 0.1–100 nmol/L, 5 minutes) (Peptides International, Inc., KY, USA) in 32.5 mmol/L KCl preconstricted vessels, in the absence or presence of 20 μmol/L L-carnitine (30 minutes). In some artery rings, the endothelium was removed by gentle abrasion of the intimal surface. Successful removal of this cell layer was determined by a reduction in the

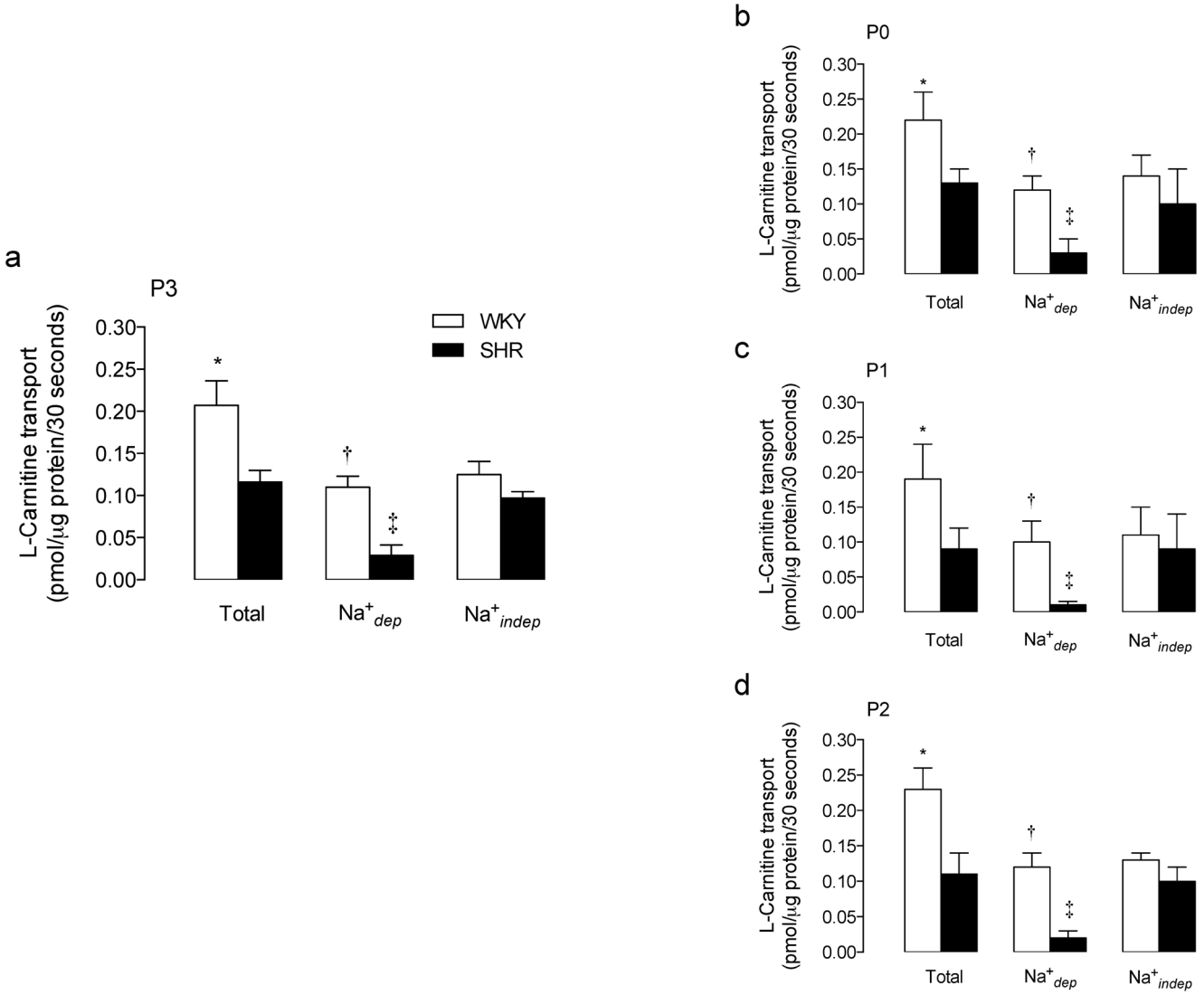

Figure 1. Transport of L-carnitine in RAECs. Total, Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) L-carnitine transport (20 μmol/L, 3 μCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) in RAECs from WKY rats or SHR. Transport was assayed in RAECs in passage 3 (P3) (a) and compared with cells in passages 0 (P0) (b), 1 (P1) (c) or 2 (P2) (d). *$P<0.05$ versus all other values, †$P<0.05$ versus corresponding Na^+_{dep} values in SHR, ‡$P<0.05$ versus all other values in SHR. Values are mean ± SEM (n = 7–20).
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vasodilatation to CGRP. Changes in isometric tension were recorded using the software LabChart (LabChart 7 for Windows, ADInstruments, Australia) coupled to a PowerLab (PowerLab 8/30 Data Acquisition System, ADIntruments, Australia). The tissue responses are as a percentage of maximal contraction induced by 62.5 mM KCl.

Statistical analysis

Values are mean ± SEM, where n indicates the number of different cell cultures (3–4 replicates). Data reported in this study describe a normal standard distribution and comparison between two or more than two groups were performed by means of Student's unpaired *t*-test and analysis of variance (2-way ANOVA), respectively. If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by the multiple-comparison Bonferroni correction test. The statistical software GraphPad Instat 3.0b and Graphpad Prism 6.0d (GraphPad Software Inc., San Diego, CA, USA) were used for data analysis. $P<0.05$ was considered statistically significant.

Results

Overall transport of L-carnitine

The *TTC* for 20 μmol/L L-carnitine was lower (44±8%) in RAECs from SHR compared with WKY rats (Fig. 1a). *TTC* exhibited a $^{TTC}Na^+_{dep}$ and a $^{TTC}Na^+_{indep}$ component in cells from both SHR and WKY rats. However, in cells from WKY rats the contribution of the $^{TTC}Na^+_{dep}$ (52±6%) and $^{TTC}Na^+_{indep}$ (60±7%) components to the *TTC* were similar ($P>0.05$), whereas, *TTC* in cells from SHR resulted from a major contribution of the $^{TTC}Na^+_{indep}$ (84±6%) compared with the $^{TTC}Na^+_{dep}$ (25±5%) component of transport (Fig. 1a). Cells in passage 0 (Fig. 1b), 1 (Fig. 1c) or 2 (Fig. 1d) exhibited similar changes in L-carnitine transport compared with cells in passage 3 (Fig. 1a).

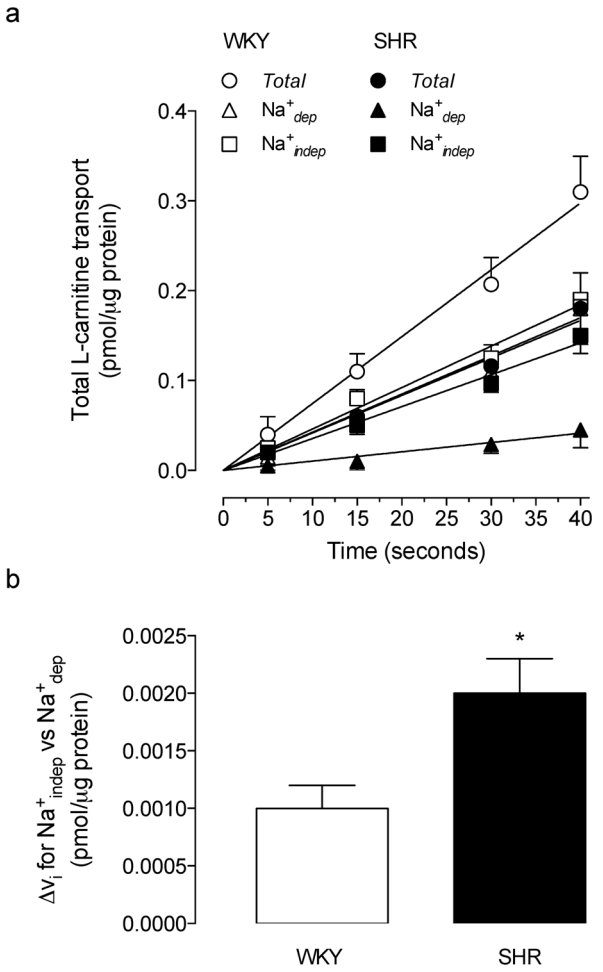

Figure 2. Initial velocities for total transport of L-carnitine. (a) Initial velocity (v_i) for total transport of L-carnitine (*Total*), and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) transport components (20 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine, 37°C) in RAECs cultured from WKY rats or SHR. (b) Difference between the v_i (Δv_i) for Na^+_{indep} and Na^+_{dep} components of transport in WKY rats or SHR. *$P<0.05$ versus WKY. Values are mean ± SEM (n = 15).
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The *TTC* was lineal up to 40 seconds incubation (Fig. 2a) with v_i values lower in SHR compared with WKY rats (Table 1). In cells from either SHR or WKY rats the v_i for the $^{TTC}Na^+_{indep}$ compared with the corresponding $^{TTC}Na^+_{dep}$ component was higher for 20 μmol/L L-carnitine transport. However, the difference between the v_i for the $^{TTC}Na^+_{indep}$ compared with $^{TTC}Na^+_{dep}$ component was higher (2.1±0.3 fold) in cells from SHR compared with WKY rats (Fig. 2b).

L-Carnitine transport kinetics

The *TTC* was semisaturable in RAECs from either SHR or WKY rats (Fig. 3a,b). The K_D value for *TTC* was similar to that for the $^{TTC}Na^+_{indep}$ component of transport in cells from WKY rats; however, the K_D for $^{TTC}Na^+_{dep}$ transport in cells from these animals and all other K_D values in SHR were negligible (Table 1). The $^{TTC}Na^+_{dep}$ and $^{TTC}Na^+_{indep}$ components derived from *TTC* were saturable in both groups of cells. The Eadie-Hofstee plot of *TTC* data was best fitted by an exponential one phase decay equation resulting in a non-linear plot in cells from WKY rats (Fig. 3c). However, the $^{TTC}Na^+_{dep}$ and $^{TTC}Na^+_{indep}$ components derived from *TTC* in WKY rats were linear (Fig. 3c), as were *TTC*, $^{TTC}Na^+_{dep}$ and $^{TTC}Na^+_{indep}$ components of transport in SHR (Fig. 3d).

After subtracting the lineal, non-saturable component of transport data (in the range of concentrations used in this study), the saturable transport for each condition was obtained (Fig. 4a,b). Cells from SHR exhibit reduced V_{max}, but unaltered apparent K_m for *TSC* compared with cells from WKY rats (Table 1). The V_{max} for the $^{TSC}Na^+_{dep}$, but not for $^{TSC}Na^+_{indep}$ components of transport was reduced in cells from SHR compared with the corresponding values in WKY rats. In addition, the V_{max} for the $^{TSC}Na^+_{indep}$ component was higher than $^{TSC}Na^+_{dep}$ component of transport only in SHR (Table 1). The Eadie-Hofstee plot of saturable transport data was lineal for all experimental conditions (Fig. 4c,d). The V_{max}/K_m values for sturable transport and the Na^+_{dep} component were lower in SHR compared with WKY rats, and the value for the $^{TSC}Na^+_{dep}$ component was lower than the $^{TSC}Na^+_{indep}$ component of transport in cells from SHR or WKY rats (Table 1).

Table 1. Kinetic parameters for transport of L-carnitine in rat aortic endothelial cells.

	L-Carnitine transport					
	WKY			**SHR**		
	Total	**Na^+_{dep}**	**Na^+_{indep}**	**Total**	**Na^+_{dep}**	**Na^+_{indep}**
TTC						
v_i	0.007±0.0001	0.003±0.002	0.004±0.0003†	0.004±0.0001*	0.001±0.0001*	0.003±0.0001†
K_D	0.0016±0.0002	$<10^{-13}$	0.0013±0.0013	$<10^{-13}$	$<10^{-15}$	$<10^{-14}$
TSC						
V_{max}	0.84±0.2	0.42±0.06	0.46±0.2	0.59±0.07*	0.20±0.03*	0.32±0.08†
K_m	28±9	46±19	21±4	30±8	31±11	22±16
V_{max}/K_m	0.030±0.008	0.009±0.002	0.022±0.009†	0.020±0.004*	0.006±0.002*	0.015±0.007†

Total (*TTC*) and saturable (*TSC*) transport of L-carnitine and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) components of transport (0–80 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C, pH 7.4) were measured in cultured (passage 2) rat aortic endothelial cells (RAECs) from normotensive (WKY) or spontaneously hypertensive (SHR) rats as described in Methods. The initial velocity (v_i) was measured for 20 μmol/L L-carnitine up to 30 seconds. v_i, initial velocity (pmol/μg protein/second); V_{max}, maximal velocity (pmol/μg protein/minute); K_m, apparent Michaelis-Menten constant (μmol/L); V_{max}/K_m, maximal transport capacity (pmol/μg protein/minute/(μmol/L)); K_D, lineal, non-saturable transport in the range of L-carnitine concentrations used in this study (pmol/μg protein/minute/(μmol/L)). *$P<0.05$ versus corresponding values in WKY, †$P<0.05$ versus corresponding values for Na^+_{dep} in SHR or WKY rats. Values are mean ± SEM (n = 15).
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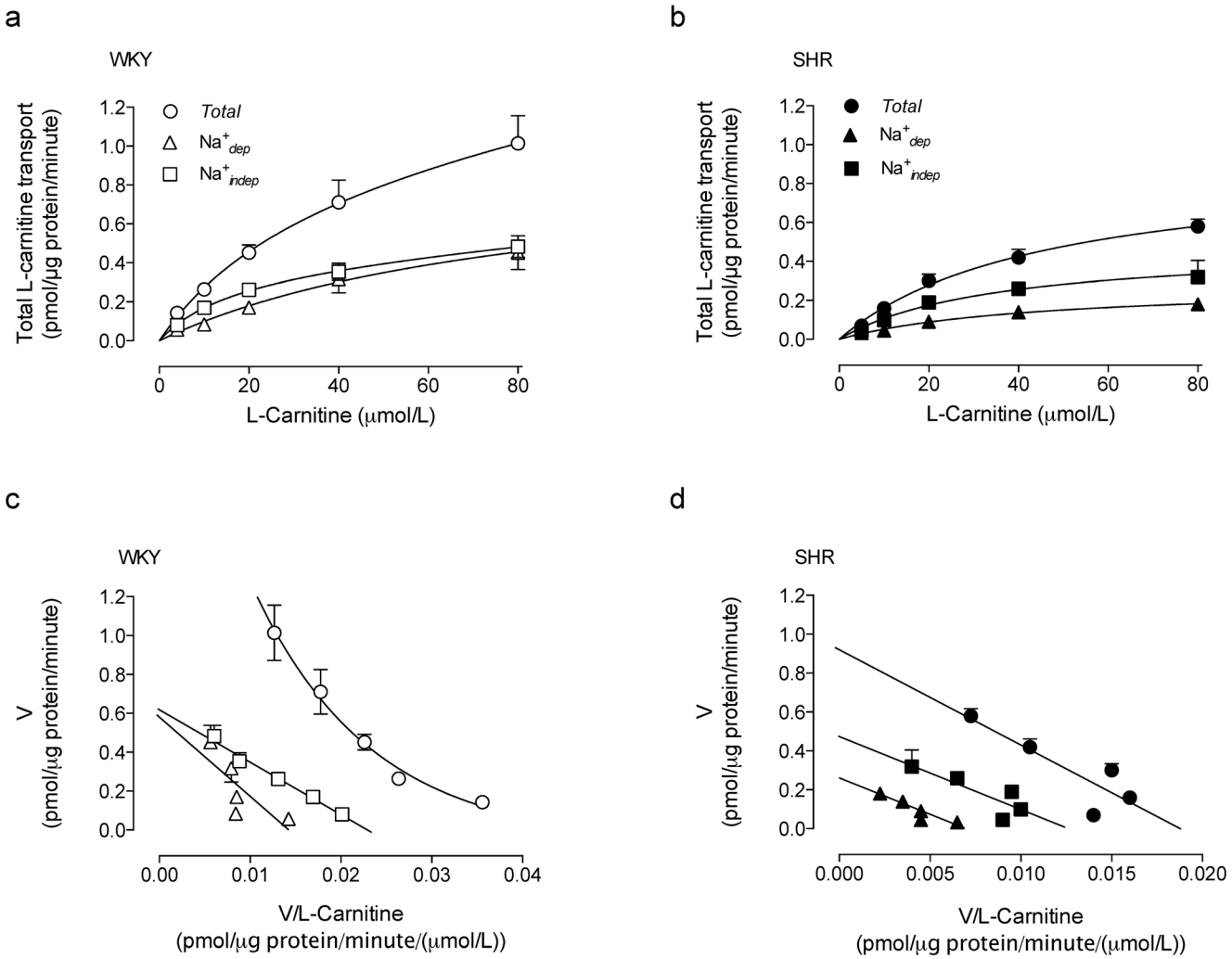

Figure 3. Total transport of L-carnitine kinetics. Total transport of L-carnitine (*Total*), and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) transport components (0–80 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) in RAECs cultures from WKY rats (a) or SHR (b). The Eadie-Hofstee plots for transport data in shown for WKY rats (c) and SHR (d) from data in (a) and (b), respectively. Values are mean ± SEM (n = 15).
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Expression of rOctn1 and rOctn2 mRNA

The relative expression of rOctn2 was higher than rOctn1 mRNA in RAECs from SHR or WKY rats (Fig. 5). The relative mRNA expression of rOctn2 was largely lower in SHR when compared with WKY; however, no differences were found in mRNA expression of rOctn1 between cells from these animals.

Extracellular pH dependency on saturable L-carnitine transport

Overall transport of L-carnitine was higher at pH_o 8.5 compared with transport at pH_o 7.4, but it was unaltered by lower pH_o values in cells from WKY rats (Fig. 6a). The $^{TSC}Na^+_{indep}$ component of transport at pH_o 7.4 was higher than at pH_o 8.5, but lower than at acidic pH_o values. However, the $^{TSC}Na^+_{dep}$ component of transport at pH_o 7.4 was lower than at alkaline, but higher than at acidic pH_o. The half-maximal stimulatory effect (SE_{50}) of a change in the pH_o on overall transport was higher than the SE_{50} for the $^{TSC}Na^+_{indep}$ (~0.26 pH_o units of difference) component (Table 2). A similar effect on *TSC* was seen for the half-maximal inhibitory effect (IE_{50}) of a change in the pH_o on the $^{TSC}Na^+_{dep}$ (~0.53 pH_o units of difference) component of transport in cells from WKY rats (Table 2). In addition, the SE_{50} for $^{TSC}Na^+_{indep}$ was significantly different from the IE_{50} for $^{TSC}Na^+_{dep}$ values (~0.24 pH_o units of difference) in these cells.

In RAECs from SHR, the *TSC* and the $^{TSC}Na^+_{indep}$ component of transport were lower at alkaline pH_o compared with transport at pH_o 7.4 (Fig. 6b). However, the $^{TSC}Na^+_{dep}$ component of saturable transport was unaltered compared with values at pH_o 7.4 in these cell types. The IE_{50} values for overall and the $^{TSC}Na^+_{indep}$ component of L-carnitine transport (~0.05 pH_o units of difference) were not significantly different in cells from SHR (Table 2). However, the alkalization required to reduce the overall transport in cells from SHR was higher (~0.22 pH_o units of difference) compared with the alkalization required to increase the transport in cells from WKY rats. In addition, the alkalization required to reduce the $^{TSC}Na^+_{indep}$ component of transport in cells from SHR was higher (~0.93 pH_o units of difference) compared with cells from WKY rats.

Rat aorta reactivity

CGRP caused dilation of preconstricted aortic rings in both group of animals (Fig. 6c). However, the half-maximal vasodilation

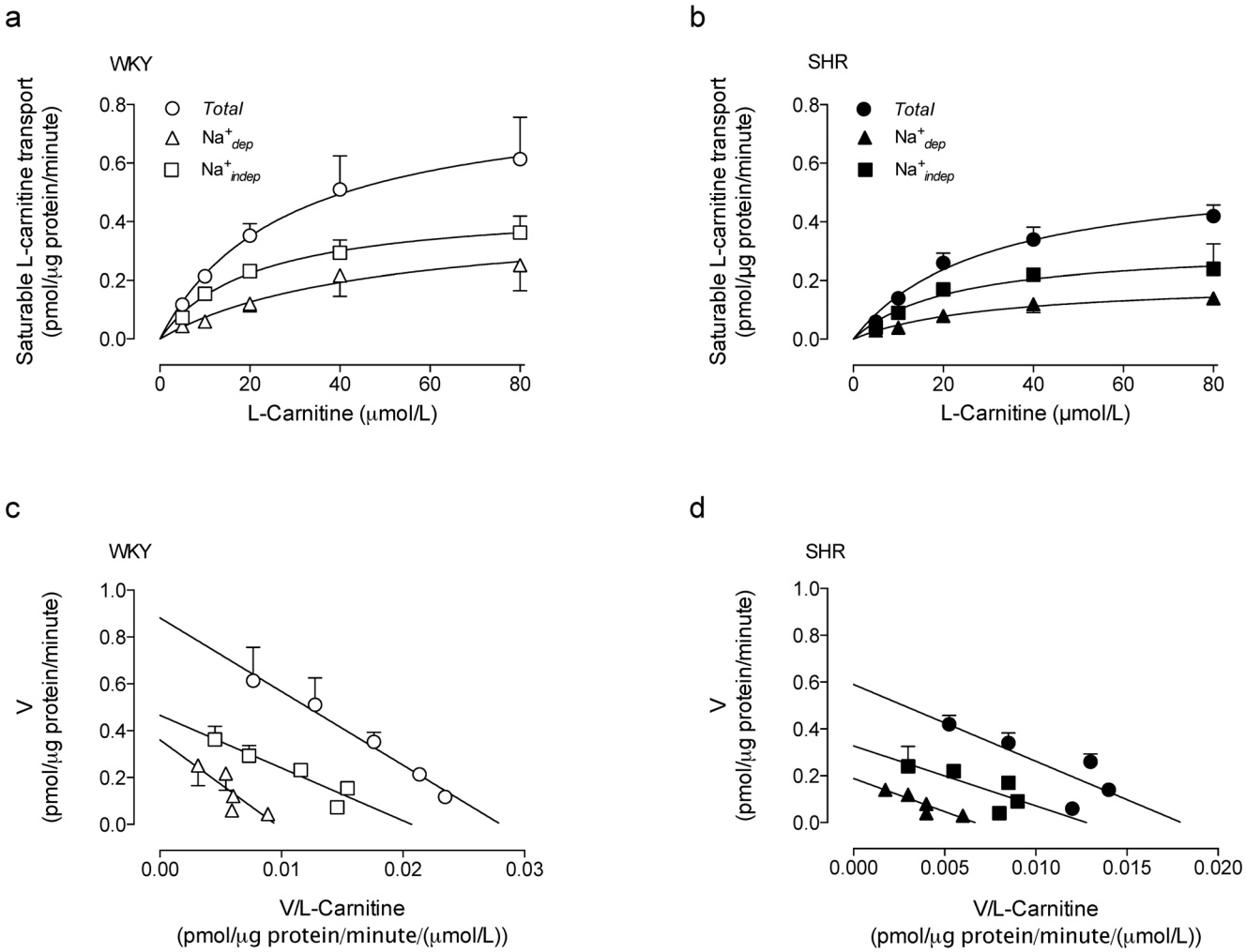

Figure 4. Saturable transport of L-carnitine kinetics. Total saturable transport of L-carnitine (*Total*), and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) transport components (0–80 μmol/L L-carnitine, 3 μCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) in RAECs cultures from WKY rats (a) or SHR (b). The Eadie-Hofstee plots for transport data are shown for WKY rats (c) and SHR (d) from data in (a) and (b), respectively. Values are mean ± SEM (n = 15).
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(EC_{50}) caused by CGRP was lower in vessels from SHR (EC_{50} = 9.5±0.3 nmol/L) compared with WKY (EC_{50} = 1.0±0.2 nmol/L) rats. Supplementation with L-carnitine caused a reduction of the EC_{50} for CGRP in vessels from SHR (EC_{50} = 1.9±0.3 nmol/L), but did not alter ($P>0.05$) this parameter in vessels from WKY rats (EC_{50} = 0.9±0.1 nmol/L). CGRP was ineffective in endothelium-denuded rat aortic rings (Fig. 6d).

Discussion

We have characterized the kinetics of L-carnitine transport in primary cultures of rat aortic endothelial cells (RAECs) from non-hypertensive WKY rats and contrasted this information with cells from spontaneously hypertensive rats (SHR). Total overall transport of L-carnitine (*TTC*) was mediated by Na^+-dependent ($^{TTC}Na^+_{dep}$) and Na^+-independent ($^{TTC}Na^+_{indep}$) components increased by a lineal, nonsaturable mediated transport. A reduced initial velocity for the *TTC* and $^{TTC}Na^+_{dep}$, but not the $^{TTC}Na^+_{indep}$ component was found in cells from SHR compared with non-hypertensive WKY rats. The kinetics assays for saturable overall transport of this amino acid (*TSC*) show that maximal transport capacity (V_{max}/K_m) for L-carnitine is lower in cells from SHR compared with WKY rats, a finding paralleled by reduced V_{max}/K_m for the $^{TSC}Na^+_{dep}$, but not the $^{TSC}Na^+_{indep}$ component. A differential dependency of pH_o for *TSC*, $^{TSC}Na^+_{dep}$ and $^{TSC}Na^+_{indep}$ transport was seen in cells from SHR compared with WKY rats. These results are the first demonstration that RAECs from SHR exhibit a phenotype characterized by reduced L-carnitine transport compared with cells from non-hypertensive rats. These results are potentially useful for a better understanding of the membrane transport mechanisms of L-carnitine in RAECs from non-hypertensive WKY rats and SHR. Interestingly, a reduced reactivity to an endothelium dependent vasodilator of the aortic rings from SHR compared with WKY rats was seen, an effect that was improved by supplementation of these vessels *in vitro* with L-carnitine. However, vasodilation was absent in endothelium-denuded aortic ring preparations. It is suggested that a reduced uptake of L-carnitine by the endothelium could counteract the reported beneficial vascular effects of L-carnitine supplementation in subjects with hypertension.

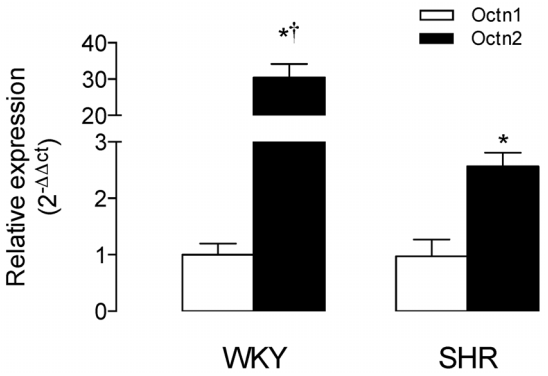

Figure 5. Expression of Octn1 and Octn2. The relative mRNA expression level of Octn1 and Octn2 in RAECs cultures from WKY rats or SHR was estimated from the $2^{-\Delta\Delta CT}$ method using the Light Cycler® 480 SW 1.5 relative quantification (delta-delta-Ct) study software as described in Methods. Gene expression levels were normalized to GAPDH mRNA level. *$P<0.05$ versus corresponding values for Octn2. †$P<0.02$ versus corresponding values in SHR. Values are mean ± SEM (n = 15).
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L-Carnitine transport in normotensive rats

Several reports describe that the natural amino acid L-carnitine could act by improving the high arterial blood pressure in patients with essential hypertension [3,4] and in animal models of hypertension [5,26]. These results complement the possibility that in hypertension the membrane transport of this amino acid is reduced, a phenomenon that is supported by studies showing a correlation between increased L-carnitine plasma level and higher blood pressure in adult men [31]. Plasma membrane transport of L-carnitine is mediated by OCTNs in mammalian cells, and the isotypes Octn1, Octn2 and Octn3 have been cloned from rats [12,32]. Our results show that RAECs exhibit *TTC* mediated by at least three different components (Na^+-dependent ($^{TTC}Na^+_{dep}$), Na^+-independent ($^{TTC}Na^+_{indep}$) and lineal, nonsaturable transport) in the range up to 80 μmol/L L-carnitine. These findings agree with the basic characteristics described for Octn-like transport of this amino acid in other cell types [33–35]. Since the Eadie-Hofstee representation of the *TTC* data was not lineal, it is likely that at least two or more transport systems acting in parallel [36,37] will account for L-carnitine transport in RAECs from non-hypertensive rats. In addition, since the $^{TTC}Na^+_{dep}$ and $^{TTC}Na^+_{indep}$ components of transport were lineal in the Eadie-Hofstee plot, either a single transport system or two or more transport systems with similar kinetic parameters acting in parallel mediate the Na^+-dependent and the Na^+-independent L-carnitine transport in this cell type.

Our results also show that cells from WKY rats exhibit a *TSC* resulting from a pronounced differential contribution of the $^{TSC}Na^+_{dep}$ and the $^{TSC}Na^+_{indep}$ components when the relative V_{max}/K_m for these components were compared. The relative contribution of the V_{max}/K_m for the $^{TSC}Na^+_{dep}$ component to *TSC* is lower (~30%) (from $1/^{TSC/Na+\text{-}dep}F = 0.30$) compared with the contribution accounted by the $^{TSC}Na^+_{indep}$ component (~73%) ($1/^{TSC/Na+\text{-}indep}F = 0.73$). Thus, a Na^+_{indep} component of L-carnitine transport predominates in RAECs from non-hypertensive WKY rats.

Octn1 is widely expressed in several tissues, including microvascular endothelium from human heart [38], and mediates L-carnitine transport via a Na^+-independent mechanism [34,39] with apparent K_m values ranging from 2–200 μmol/L [13–15]. Since the results of our study show that the apparent K_m for the $^{TSC}Na^+_{dep}$ and $^{TSC}Na^+_{indep}$ components (K_m = 21–46 μmol/L) was in the range of values described for this membrane transporter isoform in other cell types [35], the possibility that Octn1 was responsible of the Na^+-independent L-carnitine transport in RAECs from WKY rats is supported. However, Octn2-mediated transport of L-carnitine is described as a Na^+-dependent transport mechanism with higher affinity (K_m = 2–20 μmol/L) compared to Octn1 [16,18–22]. Octn2 is also expressed in other types of endothelial cells, including human heart and brain capillaries endothelium [23,40]; therefore Octn2 could also account for the Na^+-dependent transport of L-carnitine in RAECs. Interestingly, since the plasma concentration of L-carnitine for WKY rats is reported as 20–36 μmol/L [41] it is likely that the lower affinity transport system Octn1 would play a preferential role compared with Octn2, which is likely to be saturated at physiological L-carnitine plasma concentrations, in maintaining the extracellular physiological concentrations of this amino acid in these animals.

In the present study, both Octn1 and Octn2 mRNA expression was detected in RAECs from WKY rats. Interestingly, Octn2 mRNA relative expression resulted to be ~31 fold higher compared with Octn1 mRNA in these cells. Since the relative contribution of the $^{TSC}Na^+_{dep}$ component to the V_{max}/K_m for *TSC* was ~30%, it is likely that not more than ~10 fold change in Octn2 mRNA expression (estimated from the (Octn2 mRNA/Octn1 mRNA)/($1/^{TSC/Na+\text{-}dep}F$) ratio) could sustain a $^{TSC}Na^+_{dep}$ component for the *TSC* in RAECs. The remaining transport mediated via a $^{TSC}Na^+_{indep}$ component (~73%) could represents the contribution of a Na^+-independent transport activity derived from Octn1 in this cell type. Interestingly, since the contribution for the Na^+-dependent Octn2 transport is reported as ~3 fold higher than the transport detected in the absence of extracellular Na^+ in other cell types [42], an equivalent fractional contribution for these components to L-carnitine transport in RAECs from WKY rats could be expected. However, the latter seems unlikely in this cell type since the relative contribution of the $^{TSC}Na^+_{dep}$ component to the V_{max}/K_m for *TSC* was ~30% ($1/^{TSC/Na+\text{-}dep}F$ = 0.3) compared with ~73% ($1/^{TSC/Na+\text{-}indep}F$ = 0.73) for the $^{TSC}Na^+_{indep}$ component. Thus, contrasting with other cell types [35,42], these findings further support the possibility that the Na^+_{indep} component predominates (~2.4 fold) compared with the Na^+_{dep} component regarding their contribution to *TSC*. This could be interpreted as a major contribution of Octn1 compared with Octn2 to the saturable transport of L-carnitine in RAECs from WKY rats.

L-Carnitine transport in RAECs from WKY rats was also dependent on the pH_o, a characteristic well described for Octn1 [13,32] and Octn2 [43]. Our results show that *TSC* was increased by ~20% by alkalization of the extracellular medium, an effect resulting from a combined increases in the $^{TSC}Na^+_{indep}$ component (~58%) and decreases in the $^{TSC}Na^+_{dep}$ component (~29%). This phenomenon could be due to a higher sensitivity to alkalization of the $^{TSC}Na^+_{dep}$ [(*TSC* SE_{50}) minus ($^{TSC}Na^+_{dep}$ IE_{50}) = 0.26 units of pH_o] compared with the $^{TSC}Na^+_{indep}$ [(*TSC* SE_{50}) minus ($^{TSC}Na^+_{indep}$ IE_{50}) = 0.50 units of pH_o] components regarding the change seen in *TSC*. Based in these findings, a higher alkalization-dependent increase in the *TSC* could be reached whether these two transport components were equally altered or whether the $^{TSC}Na^+_{indep}$ component was unaltered by this environmental condition. Interestingly, the increase of *TSC* caused by a change of 0.6 units of pH_o in RAECs was ~3.3 fold the increase reported for 150 μmol/L tetraethylammonium (TEA) uptake in response to a similar change in pH_o units in HEK293 cells expressing the human OCTN1 form [13]. However,

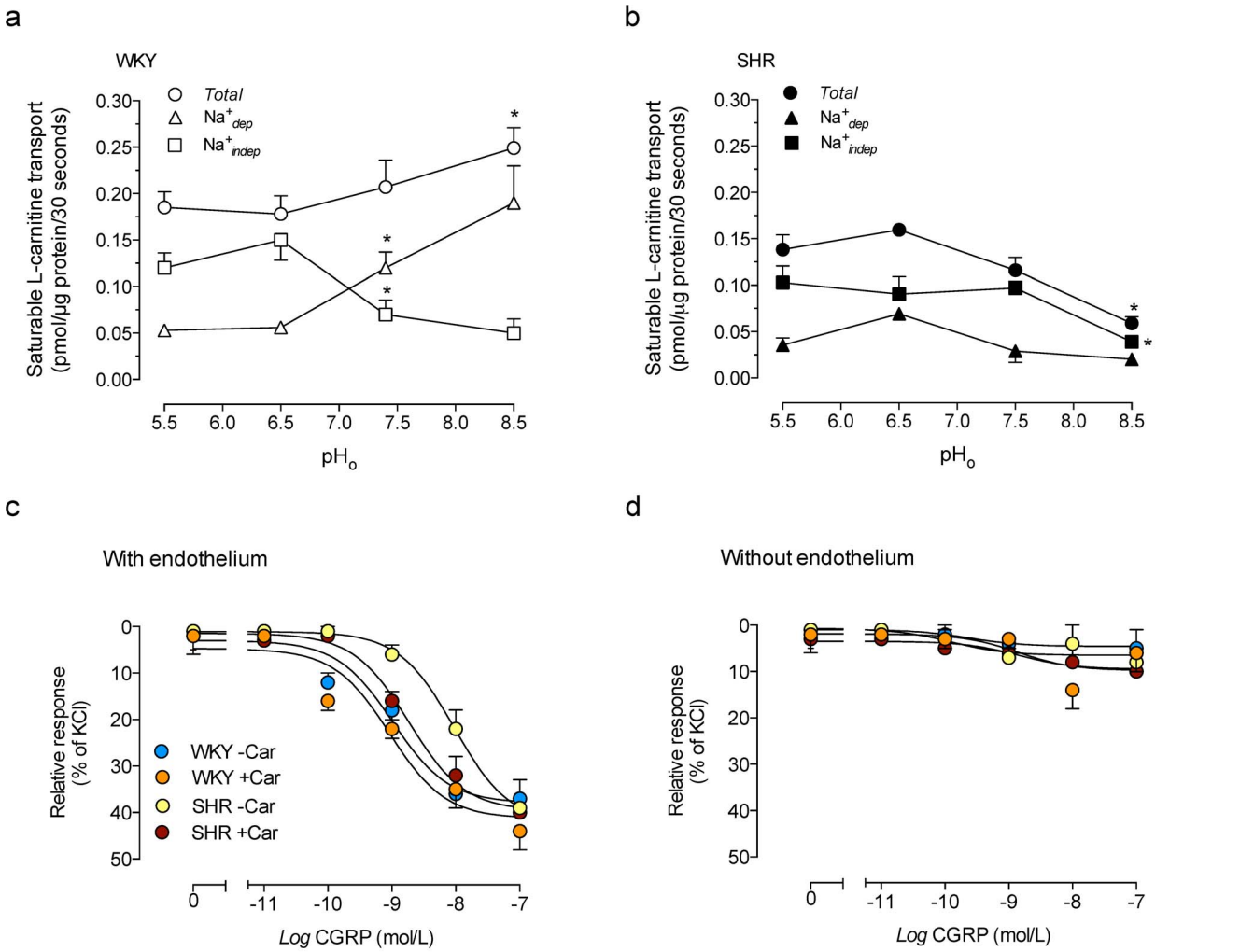

Figure 6. Effect of extracellular pH on saturable transport of L-carnitine, and rat aorta reactivity. Total saturable transport of L-carnitine (*Total*), and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) transport components (20 µmol/L L-carnitine, 3 µCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) in RAECs cultures from WKY rats (a) or SHR (b) exposed to culture medium with the pH adjusted to different values. (c) Relaxation of 32.5 mmol/L KCl preconstricted endothelium-intact aortic vessel rings (With endothelium) from WKY rats or SHR in response to increasing concentrations of calcitonine gene related peptide (CGRP, 5 minutes), in the absence (-Car) or presence (+Car) of 20 µmol/L L-carnitine (30 minutes). (d) Relaxation of endothelium-denuded aortic vessel rings (Without endothelium) to CGRP as in (c). *$P<0.05$ versus all other values for the corresponding components. Values are mean ± SEM (n = 4–10).
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contrasting with these results a larger increase in the pH_o value (1 unit of pH_o) reduced overall transport of TEA in these cells [32]. Thus, it is likely that OCTNs-like transport is differentially responsive to the degree of alkalization reached in HEK293 cells. Interestingly, a change in ~0.4 units of pH_o has been shown to increase the activity of other membrane transport systems, such as the sodium/proton exchanger isoform 1 in MDCK cells [44], suggesting that modulation of Octn1/2 by a similar change in the pH_o in RAECs agree with what is reported in other cell types. On the other hand, acidification of the extracellular pH_o does not alter *TSC*, a net effect that result from a proportional reduced $^{TSC}Na^+_{dep}$ and increased $^{TSC}Na^+_{indep}$ transport components. Interestingly the effect of extracellular acidification was similar for both components, suggesting that these components are equally sensitive to acidification in RAECs from WKY rats. Thus, acidification and alkalization of extracellular medium results in a differential modulation of L-carnitine transport in RAECs from non-hypertensive rats.

Effect of spontaneous hypertension on L-carnitine transport

Cells from SHR exhibit a semisaturable *TTC* unaffected by a lineal, non-saturable component in the range of L-carnitine concentrations used in this study. This data was best fitted to a first-order regression line in an Eadie-Hofstee plot, suggesting that one or more transport systems with apparent K_m values in the same range could mediate L-carnitine transport in RAECs from SHR. The apparent K_m values for L-carnitine transport by RAECs in SHR were similar to values detected in non-hypertensive rats, and within the range of the plasma L-carnitine concentration reported in SHR (21–41 µmol/L) [41]. Thus, L-carnitine transport via Octn1/2 could contribute to maintain the physiological plasma concentration of this amino acid in SHR. Our results also show that *TTC* is mainly mediated by a $^{TTC}Na^+_{indep}$ (~75%) with a minor contribution of $^{TTC}Na^+_{dep}$ (~25%) components. These findings agree with those obtained by contrasting the relative V_{max}/K_m for these components with that for *TSC*. Since

Table 2. Half-maximal effect of extracelular pH on saturable transport of L-carnitine in RAECs.

	SE_{50} (pH_o units)	IE_{50} (pH_o units)
WKY		
Total	7.73±0.25	*ni*
Na^+_{dep}	7.47±0.11*	*ni*
Na^+_{indep}	*ns*	7.23±0.14
SHR		
Total	*ns*	7.95±0.13
Na^+_{dep}	*ns*	*ni*
Na^+_{indep}	*ns*	8.10±0.09

Total transport of L-carnitine, and the Na^+-dependent (Na^+_{dep}) and Na^+-independent (Na^+_{indep}) transport components (20 µmol/L L-carnitine, 3 µCi/mL L-[^{3}H]carnitine, 30 seconds, 37°C) in RAECs cultures from WKY rats or SHR exposed to culture medium with the pH adjusted to different values (extracellular pH (pH_o) 5.5–8.5) as described in Methods. The stimulatory (SE_{50}) or inhibitory (IE_{50}) effect of pH_o on transport was calculated. *ni*, not inhibited; *ns*, not stimulated. *$P<0.05$ versus Total in WKY rats. Values are mean ± SEM (n = 12).

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the relative contribution of the V_{max}/K_m for the $^{TSC}Na^+_{indep}$ component is higher (~75%) ($1/^{TSC/Na+\text{-}indep}F = 0.75$) compared with the contribution accounted by the $^{TSC}Na^+_{dep}$ component (~30%) ($1/^{TSC/Na+\text{-}dep}F = 0.30$), and considering that similar findings were found for the v_i values for these transport components, it is suggested that RAECs from SHR exhibit saturable transport of L-carnitine where the Na^+-independent transport predominates by ~2.5 fold compared with the Na^+-dependent. This result is ~2.1 fold higher compared with cells from non-hypertensive rats supporting the possibility that hypertension could associate with a higher requirement of Na^+-independent transport of L-carnitine via Octn1/2 activity in RAECs. Thus, it is likely that a deficiency in the $^{TSC}Na^+_{dep}$ component results in RAECs dysfunction in SHR. This would not be explained by a lower V_{max}/K_m of the $^{TSC}Na^+_{dep}$ component, since the relative contribution of this component to *TSC* in these cells was similar to that in cells from non-hypertensive rat ($(1/^{TSC/Na+\text{-}dep}F$ in WKY rats)/($1/^{TSC/Na+\text{-}dep}F$ in SHR = 1.01). In addition, the relative contribution of the $^{TSC}Na^+_{dep}$ component compared with the $^{TSC}Na^+_{indep}$ component to *TSC* in SHR is also similar to non-hypertensive rats ($(1/^{Na+\text{-}indep/Na+\text{-}dep}F$ in WKY rats)/($1/^{Na+\text{-}indep/Na+\text{-}dep}F$ in SHR = 1.03). Thus, reduced overall transport of L-carnitine in RAECs from SHR could be mainly due to reduced expression of the Na^+-dependent Octn2 and in a less extend to a reduced expression of the Na^+-independent Octn1 membrane transporters. In fact, the Octn2 mRNA expression in cells from SHR is only 2.6 fold compared with Octn1 mRNA expression, a value that is largely minor compared with the 31 fold increase for this mRNA detected in cells from non-hypertensive rats. Thus, a reduced Octn2 expression without alterations in the V_{max}/K_m could account for the reduced $^{TSC}Na^+_{dep}$ component of L-carnitine transport in RAECs from SHR.

Interestingly, as found in cells from non-hypertensive rats, the relative contribution of the $^{TSC}Na^+_{dep}$ component to the V_{max}/K_m for *TSC* was ~30%. Thus, a proportional change by Octn2 expression to (i.e., ~1.48 fold) would sustain the $^{TSC}Na^+_{dep}$ component of the saturable transport activity in RAECs from SHR. Interestingly, this value is ~85% lower compared with the potential requested change in Octn2 mRNA expression in cells from non-hypertensive rats. Therefore, SHR is a pathological condition that results in lower request of Octn2 mRNA expression compared with RAECs from non-hypertensive rats. The results also show that L-carnitine transport in RAECs from SHR was dependent on the pH_o, supporting the possibility that transport was mediated by Octn1/2 in this cell type. In this case, alkalization of the extracellular medium resulted in reduced *TSC*, which was due to reduced Na^+_{indep} component. This finding is different from RAECs from non-hypertensive rats, suggesting that alkalization could result in a differential down-regulation of L-carnitine transport in RAECs from SHR compared with non-hypertensive rats. However, since the Na^+_{indep} component of transport was also reduced in cells from non-hypertensive rats, it is likely that sensitivity of this component to a change in the pH_o is similar in cells from SHR and WKY rats.

In conclusion, the kinetic parameters of L-carnitine transport in RAECs from SHR and non-hypertensive WKY rats were characterized. The overall saturable transport was mediated by Na^+_{indep} and Na^+_{dep} components, with the latter being crucial in the reduced maximal transport capacity detected in cells from SHR. The kinetic parameters, pH_o- and Na^+-dependency of transport suggest that Octn1 and Octn2 are likely responsible for membrane transport of L-carnitine in endothelial cells from the aorta of SHR and WKY rats. These results are the first characterizing the kinetic parameters for the membrane transport mechanisms of L-carnitine in rat aortic endothelium from non-hypertensive WKY and spontaneously hypertensive rats. Furthermore, since (a) the reactivity of aortic rings to the endothelium-dependent vasodilator CGRP was reduced in preparations from SHR compared with WKY rats, (b) L-carnitine supplementation *in vitro* restored CGRP vasodilation to values in vessels from normotensive rats, and (c) CGRP was ineffective in endothelium-denuded rat aortic rings, it is suggested that restoration of a functional endothelium could result from bioavailability of L-carnitine to the aorta endothelium in the spontaneously hypertensive animals.

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Author Contributions

Conceived and designed the experiments: AM CV LS. Performed the experiments: EG-G RS CS FP SZ AJB MVR-A PA AL. Analyzed the data: EG-G RS AL AM CV LS. Contributed reagents/materials/analysis tools: AL CV LS. Wrote the paper: RS EG-G CV LS.

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