Beneficial effect of walnuts on vascular tone is associated with Akt signalling, voltage-dependent calcium channel LTCC and ATP-sensitive potassium channel Kv1.2

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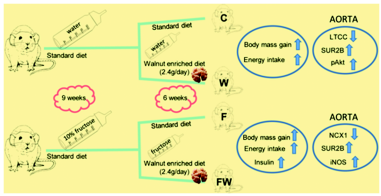
ABSTRACT

Consumption of walnuts is beneficial for cardiovascular health. To study walnut effects on proteins involved in vascular tone regulation, control and fructose-fed rats were subjected to walnut diet for 6 weeks. In contrast with increased energy intake and body mass gain, aortic protein level of L-type calcium channel alpha subunit was decreased and the level of SUR2B subunit of ATP-sensitive K + channel was increased in healthy rats subjected to walnuts, together with improved Akt phosphorylation. Upon the walnut diet in rats subjected to fructose overload, the rise in energy intake and body mass gain, was followed by an increase in blood insulin. Although SUR2B level was elevated, the level of sodium-calcium exchanger NCX1 and inducible nitric oxide synthase were reduced and increased, respectively. In summary, walnut consumption was accompanied with moderate beneficial vascular effect in healthy rats, while an effect of walnut in rats with metabolic disturbances was rather controversial.



Keywords: Walnuts ; fructose-rich diet ; vascular tone ; ion channels ; nitric oxide ; protein kinase

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Introduction

Walnut (*Juglans regia*), a constituent of traditional human diet, contains numerous bioactive compounds (Croitoru et al. 2019). Its consumption has been shown to be associated with lowering of cardiovascular risk, probably due to an improvement of lipid profile (Guasch-Ferré et al. 2018) and endothelial function (Mohammadi-Sartang et al. 2018). On the other hand, it is well documented that high intake of fructose is accompanied with metabolic syndrome features (Hannou et al. 2018). Studies on walnut effects in metabolic syndrome are quite rare (Aronis et al. 2012; Scott et al. 2017). Regarding the fact that one of the mediators of detrimental effects of fructose-rich diet (FRD) is an increase in circulating uric acid, it is interesting to mention, that two peptides isolated from walnut shown antihyperuremic effect (Li et al. 2018).

Vascular tone plays an important role in the regulation of blood pressure and the distribution of blood flow throughout the body. Regulation of the contractile activity of vascular smooth muscle cells (VSMC) is dependent on the interactions between vasodilator and vasoconstrictor stimuli (Jackson 2000). Ion channels play a crucial role in many vasculature regulatory processes, such as the cell membrane potential and vasomotor functions (Cheng et al. 2019).

Ca^{2+} channels open in response to vasoconstrictor stimuli, leading to Ca^{2+} influx and VSMC contraction (Cheng et al. 2019). Voltage-gated L-type Ca^{2+} channels (LTCC) has been recognised as an important player in the vascular tone regulation (Moosmang et al. 2003). Plasma membrane Na^{+}/Ca^{2+} exchanger (NCX1), which moves Na^{+} in and Ca^{2+} out of the VSMC is also involved in regulating Ca^{2+} homeostasis of blood vessels (Zhang et al. 2005). In opposite, K^{+} channels play an important role in the regulation of VSMC relaxation. The opening of K^{+} channels in response to membrane depolarisation or intracellular Ca^{2+} rise, results in K^{+} efflux from VSMC, leading to hyperpolarization of the plasma membrane, closing of Ca^{2+} channels, a decrease in intracellular Ca^{2+} levels, and vasodilation (Cheng et al. 2019). The ATP-sensitive K^{+} (KATP) channels play an important role in the regulation of resting membrane potential and tone of arteriolar muscle cells (Jackson 2000). The major vascular isoform of KATP channels is composed of Kir6.1 and SUR2B subunits (Shi et al. 2012). On the other hand, MaxiK channel in VSMC functions as a fine adjuster of the membrane potential and the intracellular Ca^{2+} concentrations, associated with VSMC membrane hyperpolarization and relaxation (Tanaka et al. 2004).

Nitric oxide (NO) is a potent vasodilator, involved in the relaxation of VSMC. Endothelial NO synthase (eNOS) is principally responsible for NO production in vasculature, while inducible NOS (iNOS) is upregulated during inflammation (Tejero et al. 2019).

Vascular tone regulation is also mediated by various protein kinases. Protein kinase B/Akt kinase directly activates eNOS by phosphorylation at Ser^{1177}, leading to the vasorelaxation (Michell et al. 1999). Besides that, AMP-activated protein kinase (AMPK) is involved in the relaxation of VSMC by regulation of actin depolymerisation (Schubert et al. 2017). In contrast, Ca^{2+}-dependent extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation leads to the contraction of VSMC and subsequent vasoconstriction (Palen et al. 2005).

The mechanism responsible for endothelial effects of walnuts, vascular tone and blood pressure regulation, are not completely elucidated yet. Therefore, in this study we aimed to add knowledge on the molecular mechanisms responsible for walnut effects directed to vascular tone regulation.

Materials and methods

Materials

Fructose was purchased from Omnia Nisasta Sanayi ve Ticaret A.S. (Adana, Turkey). Antibodies raised against: LTCCα, NCX1, Kir6.1, SUR2B, MaxiKα, phospho Ser^{473} Akt, phospho Thr^{172} AMPK, Akt, AMPK and β-actin, as well as secondary horse radish peroxidase (HRP)-conjugated anti-rabbit, anti-goat and anti-mouse antibodies, were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Antibodies against iNOS and eNOS are products of BD Biosciences (Franklin Lakes, USA). Anti-phospho p44/42 ERK1/2 (Thr^{202}/Tyr^{204}) and anti-p44/42 ERK1/2 were obtained from Cell Signalling Technology Inc. (Danvers, MA, USA). Reagents for the bicinchoninic acid (BCA) assay were purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Electrophoretic reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Animals and treatment

Thirty-six male Wistar rats, 21-days-old, were randomly divided into two experimental groups: control (C) and fructose~~-rich diet~~ (F) group. All rats were fed *ad libitum* a standard laboratory food, but instead of tap water, F group animals drank tap water supplemented with 10% fructose. After 9 weeks, both groups were additionally divided into two subgroups. Diet for half of C and half of F rats were supplemented with 2.4 g of walnut kernels per day during the next six weeks (W and FW group, respectively). Thereafter, animals were sacrificed, and blood and aorta samples were collected.

Standard food and liquid consumption were recorded daily. Energy intake was calculated as a sum of kJ originated from the standard food, fructose solution and walnut kernels. Body mass was recorded weekly and visceral adipose tissue mass was determined at the sacrifice.

All animal procedures and protocols were conducted in accordance with the EU Directive (2010/63/EU) on the protection of animals used for experimental and other scientific purposes and were approved by the official Vinca Institute's Ethical Committee for Experimental Animals.

Blood plasma preparation and biochemical measurements

Before measurement of biochemical parameters, rats were fasted overnight. Blood was collected in anticoagulant-coated tubes at decapitation and plasma samples were obtained by centrifugation. The glucose concentration was measured in whole blood using Accutrend glucometer (Roche Diagnostics GmbH, Mannheim, Germany). The insulin concentration was determined in plasma, commercially by the RIA method (INEP, Belgrade, Serbia).

Aortic protein isolation

Rat aorta samples were homogenised 3 × 30 s at 4 °C with a glass-glass homogeniser in modified RIPA buffer (50 mmol/l Tris-HCl, pH 7.4, 150 mmol/l NaCl, 1% Triton X-100, 0.2% Na-deoxycholate, 0.2% SDS, 1 mmol/l EDTA, protease inhibitors, phosphatase inhibitors). The homogenate was centrifuged 30 min at 15,000 × g, and the supernatant was reffered to as cell lysate. After protein concentration assessment by BCA method, samples were prepared for Western blot.

SDS polyacrylamide electrophoresis and Western blot

Proteins were separated on 7.5 or 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Laemmli 1970). Membranes were blocked with 5% BSA and blotted with an antibody against: LTCCα, NCX1, Kir6.1, SUR2B, MaxiKα subunit, eNOS, iNOS, phospho Ser^{473} Akt, phospho Thr^{202}/Tyr^{204} ERK1/2 or phospho Thr^{172} AMPK. After extensive washing, membranes were incubated with the secondary HRP-conjugated antibody and used for detection with enhanced chemiluminescent substrates. After blotting with antibodies against phosphoforms of Akt, ERK1/2 and AMPK, membranes were stripped and total amount of the same protein was determined. To ensure equal protein loading in lysate samples, blots were stripped and reprobed with the β-actin antibody. Films were scanned and analysed using ImageJ software (NIH, USA). Lysate protein level was normalised to actin level, while the level of phosphorylated forms of protein kinases was expressed as ratio phospho/total kinase level.

Statistics

All data are presented as means ± SD. Statistical comparisons were performed by two-way ANOVA with a Tukey's test for post-hoc comparison using STATISTICA software (StatSoft Inc., Tulsa, OK). A value of $p < 0.05$ was considered statistically significant.

Results

Feeding behaviour and metabolic parameters

Data concerning feeding behaviour and metabolic parameters are presented in the Table 1. According to two-way ANOVA, main effect of fructose was observed related to food ($F_{(1,8)}$=55.550, $p < 0.001$) and liquid intake

($F_{(1,8)}$=23.041, $p < 0.01$). Consumption of the standard food was decreased in fructose-fed rats compared to control and walnut groups ($p < 0.01$ for F vs. C and F vs. W). Also, rats that drank fructose and ate walnuts, consumed significantly less standard food than rats from C and W group ($p < 0.01$ for both). Regarding liquid intake, both fructose groups drank significantly more liquid than control and walnut group ($p < 0.05$ for all: F vs. C, F vs. W, FW vs. C and FW vs. W). On the other hand, both factors, FRD ($F_{(1,8)}$=132.060, $p < 0.001$) and walnut diet ($F_{(1,8)}$=24.899, $p < 0.01$), affected energy intake. Rats from W group and F group had increased energy intake compared to control group ($p < 0.05$ and $p < 0.001$, respectively). Energy intake was higher in F vs. W group ($p < 0.01$). Combined treatment (FW) elevated energy intake above values in all other groups ($p < 0.001$ for FW vs. C and FW vs. W; $p < 0.05$, WFW vs. F).

Table 1. Feeding behaviour and metabolic parameters.

	Control (C)	Walnuts (W)	Fructose (F)	Fructose + Walnuts (FW)	Two-way ANOVA		
					WD	FRD	WD × FRD
Food intake (g/day/rat)	22.79 ± 0.32	23.19 ± 0.88	18.51 ± 1.66**,##	18.98 ± 0.49**,##	NS	<0.001	NS
Liquid intake (ml day/rat)	45.16 ± 1.35	45.53 ± 0.88	68.73 ± 13.63*,#	68.63 ± 9.76*,#	NS	<0.01	NS
Total energy intake (kJ/day/rat)	250.65 ± 3.55	281.43 ± 9.72*	321.85 ± 5.29***,##	353.12 ± 18.13***,$,###	<0.01	<0.001	NS
Body mass gain (g)	413.44 ± 32.15	461 ± 34.64**	439.11 ± 28.58	490.75 ± 8.61***,$$	<0.001	<0.01	NS
VAT mass/Body mass (×100)	2.72 ± 0.53	3.85 ± 0.94	3.47 ± 0.67	5.05 ± 1.14***,$$	<0.001	<0.01	NS
Glucose (mmol/l)	5.21 ± 0.41	5.20 ± 0.39	5.36 ± 0.21	5.30 ± 0.41	NS	NS	NS
Insulin (mIU/l)	15.06 ± 4.62	21.74 ± 4.50	21.84 ± 3.94	30.07 ± 7.43***,$,#	<0.001	<0.001	NS

Abbreviations: VAT: visceral adipose tissue; WD: walnuts diet; FRD: fructose rich diet; NS: not significant.

Significantly different from Control*, Walnuts#, Fructose$.

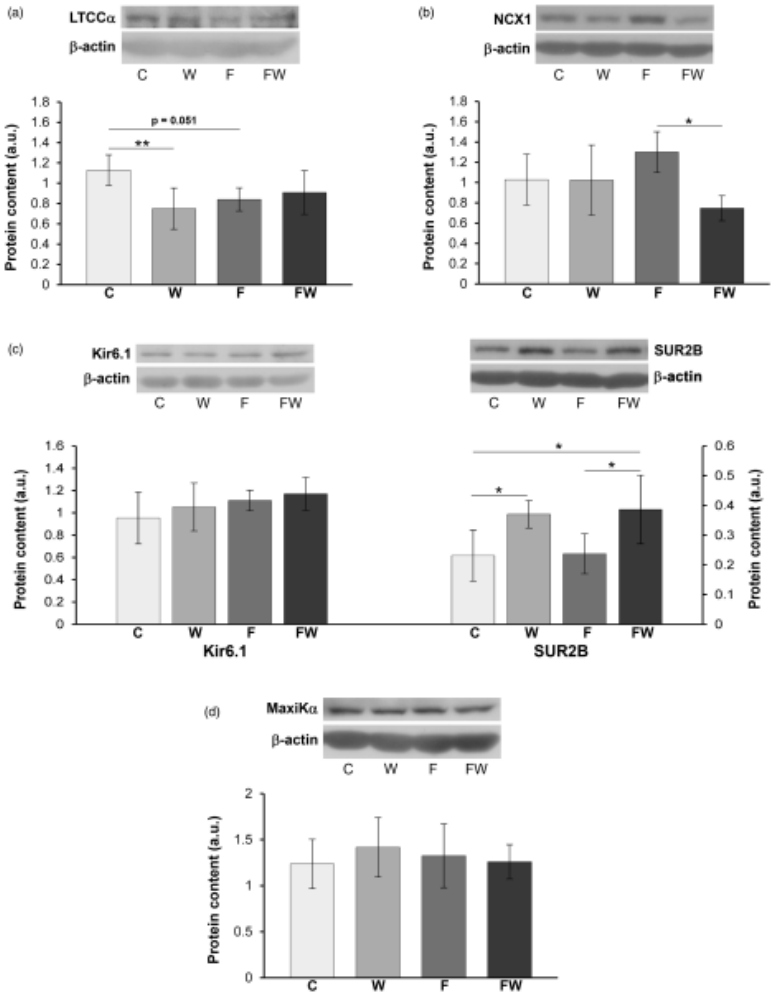
Significance is: *,#,$ $p < 0.05$; **,##,$$ $p < 0.01$; ***,### $p < 0.001$.

Two-way ANOVA detected main effects of both factors FRD ($F_{(1,31)}$=8.334, $p < 0.01$) and walnut diet ($F_{(1,31)}$=26.703, $p < 0.001$), related to body mass gain during the treatment. Rats from walnut group had significantly higher body mass gain than control rats ($p < 0.01$). Besides that, combined treatment increased body mass gain ($p < 0.001$, FW vs. C; $p < 0.01$, FW vs. F). Regarding relative visceral adipose tissue (VAT) mass, FRD ($F_{(1,26)}$=9.881, $p < 0.01$) and walnut diet effects ($F_{(1,26)}$=19.326, $p < 0.001$) were observed. Post-hoc test revealed significant increase of relative VAT mass in FW vs. C group ($p < 0.001$), and FW vs. F group ($p < 0.01$), while p value for W vs. C and FW vs. W were 0.081 and 0.059, respectively. Neither FRD, nor walnut diet (including its combination) influenced blood glucose concentration. Finally, main effect of FRD ($F_{(1,26)}$=15.505, $p < 0.001$) and walnut diet ($F_{(1,26)}$=15.076, $p < 0.001$) was observed related to blood insulin concentration. Tendency of increase the insulin concentration was detected in rats from W and F groups compared to control rats ($p = 0.076$ and $p = 0.084$, respectively). Combined treatment (FW) significantly raised insulin level above the value in all other groups ($p < 0.001$, FW vs. C; $p < 0.05$ for FW vs. W and FW vs. F).

Plasma membrane $Ca^{2+}$and K^+ ion channels

Regarding alpha subunit of LTCC, two-way ANOVA detected interaction between FRD and walnut diet ($F_{(1,20)}$=9.348, $p < 0.01$). Walnut diet affected aortic LTCC content in healthy, but not in the rats subjected to fructose overload (Figure 1(a)). In comparison to C group LTCC protein level was lower in W group ($p < 0.01$), while p value for F vs. C group decrease was 0.051. Relating to the aortic Ca^{2+}/Na^{+} exchanger, NCX1, statistical analysis also revealed interaction between treatments ($F_{(1,13)}$=4.946 $p < 0.05$) (Figure 1(b)). However, post-hoc test indicated only significant decrease in FW vs. F group ($p < 0.05$).

Figure 1. Ca^{2+} and K^{+} ion channels. The protein levels of calcium and potassium ion handling proteins (LTCCa, NCX1, Kir6.1/SUR2B, MaxiKα) were determined in aortic lysate by Western blot. Results were normalised to the actin content and are presented as mean ± S.D. Abbreviations: LTCC-L type Ca^{2+} channel; NCX1-Na^{+}/Ca^{2+} exchanger 1; C: control; W: walnuts; F: fructose rich diet; *$p < 0.05$.

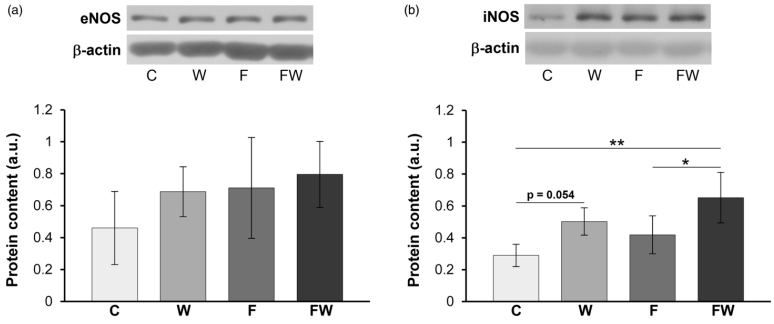


Neither main effects of separate treatments nor interactions between FRD and walnut diet were observed in the case of Kir6.1 subunit level of KATP (Figure 1(c)). Two-way ANOVA identified only statistical tendency ($F_{(1,20)}$=3.466, $p = 0.07$) regarding fructose effect. In contrast, statistical analysis revealed walnut effect on the other KATP subunit, SUR2B ($F_{(1,21)}$=17.355, $p < 0.001$) (Figure 1(c)). SUR2B level was significantly elevated in W group vs. C ($p < 0.05$). Post-hoc test also detected significant increase of SUR2B in FW group vs. C and FW vs. F group ($p < 0.05$ for both). In addition, there were no significant effects of applied treatment on aortic MaxiK channel (Figure 1(d)).

NO production enzymes

Two-way ANOVA revealed that FRD tended to affect eNOS protein expression ($F_{(1,18)}$=3.372, $p = 0.083$) (Figure 2(a)). On the other hand, concerning aortic iNOS level, analysis indicated main effects of both individual treatments, walnuts ($F_{(1,16)}$=17.426, $p < 0.001$) and FRD ($F_{(1,16)}$=6.811, $p < 0.05$) (Figure 2(b)). According to the post-hoc test, iNOS level was elevated in FW group vs. C ($p < 0.01$), FW vs. F ($p < 0.05$), and borderline elevated in W vs. C group ($p = 0.054$).

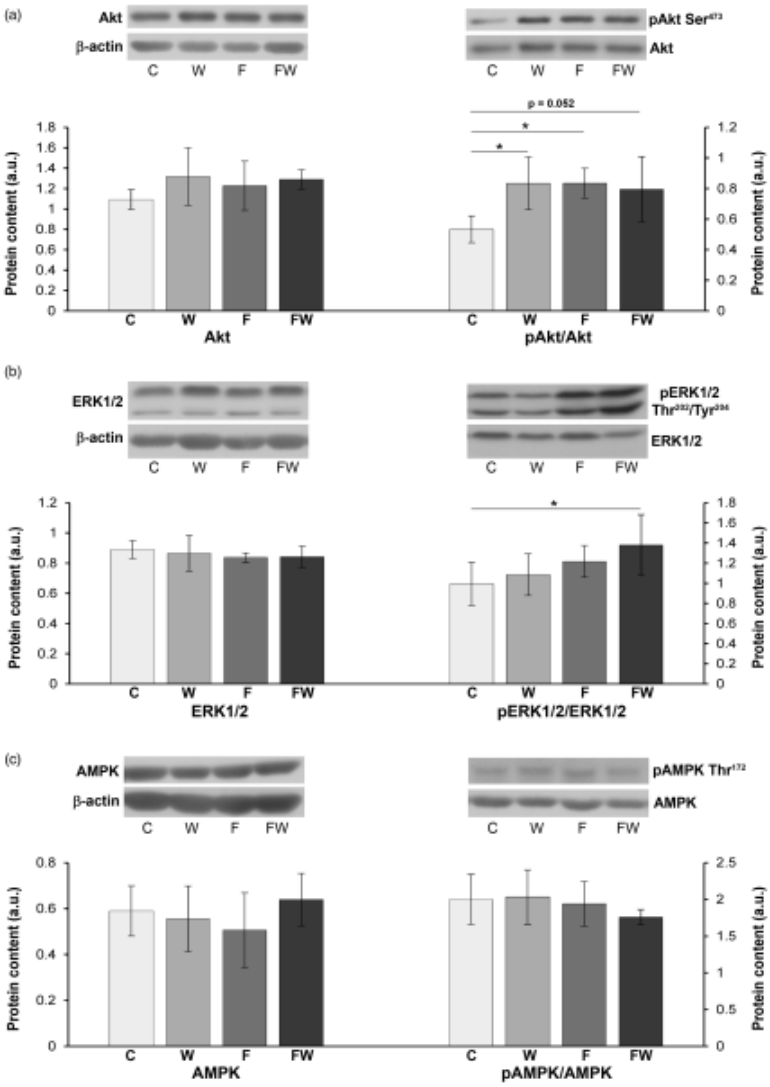
Figure 2. NO production enzymes. The protein levels of endothelial and inducible nitric oxide synthase (eNOS and iNOS, respectively) were determined in aortic lysate by Western blot. Results were normalised to the actin content and are presented as mean ± S.D. *$p < 0.05$; **$p < 0.01$. Abbreviations as in Figure 1.



Protein kinases

FRD and walnut consumption, separately or in combination, did not change Akt protein level (Figure 3(a)). However, in the case of phosphorylated Akt, two-way ANOVA indicated significant interaction between FRD and walnuts ($F_{(1,20)}$=6.498, $p < 0.05$). Both treatments significantly increased level of Akt phosphorylated at Ser^{473} ($p < 0.05$ for both, W vs. C and F vs. C), while combined treatment showed tendency to increase pAkt above control ($p = 0.052$).

Figire 3. Protein kinase signalling. The protein levels of protein kinase B/Akt, extracellular signal-regulated kinases 1 and 2 (ERK1/2) and AMP-activated protein kinase (AMPK) were determined in aortic lysate by Western blot. Results were normalised to actin content and are presented as mean ± S.D. The level of phosphorylated Akt (Ser^{473}), ERK1/2 (Thr^{202}/Tyr^{204}) and AMPK (Thr^{172}) was expressed as phospho/total kinase level. *$p < 0.05$. Abbreviations as in Figure 1.



No effect of applied treatments on ERK1/2 levels in aorta of male rats was observed (Figure 3(b)). In contrast, the main effect of FRD ($F_{(1,17)}$=5.962, $p < 0.05$) was revealed regarding ERK1/2 phosphorylation (Thr^{202}/Tyr^{204}). Tukey's test detected significant increase of ERK1/2 phosphorylation in FW vs. C group ($p < 0.05$) (Figure 3(b)).

Neither AMPK level nor its phosphorylation at Thr^{172} was altered by applied treatments (Figure 3(c)).

Discussion

Lifestyle modifications, particularly those concerning diet, are a promising approach for the prevention and treatment of the cardiovascular diseases. Walnut, traditional ingredient of human diets worldwide, contains high omega-3 fatty acid content of α-linolenic acid (ALA) that have been suggested to improve cardiovascular health (Aronis et al. 2012; Caligiuri et al. 2014; Kris-Etherton 2014). Other important components of walnuts, including dietary fibres, polyphenols, tocopherol, antioxidants, magnesium, folic acid, L-arginine and phytochemicals could also beneficially influence cardiovascular risk (Berryman et al. 2013; Mohammadi-Sartang et al. 2018).

The literature consistently confirms improvement of endothelial function associated with walnut consumption (Ma et al. 2010; Berryman et al. 2013; Mohammadi-Sartang et al. 2018; Xiao et al. 2018), but the precise mechanism has not been established. Data on the effects of walnuts on blood pressure indicate either decrease or no changes in blood pressure in patients with metabolic syndrome (Scott et al. 2017). Regarding the mechanism, walnut - induced vasodilation has been connected with reduced level of vascular cell adhesion molecule-1 in hypercholesterolemic patients

(Ros et al. 2004) and aortic endothelin-1 mRNA level decline (Davis et al. 2006). In addition, linoleic acid (LA), a component of walnuts, induces relaxation and hyperpolarization of porcine coronary VSMC via a mechanism that involves activation of the Na^+/K^+-ATPase pump (Pomposiello et al. 1998).

Results of the present study shed new light on molecular mechanism of the beneficial effects of walnuts' consumption on vascular tone. We demonstrated that in healthy rats, despite an increase in energy intake and body mass gain, walnut consumption increased the level of aortic pAkt, decreased level of LTCC alpha and elevated level of SUR2B subunit of KATP, which suggests a consistent beneficial vasodilatory effect. However, in fructose-fed rats, a favourable increase of SUR2B in walnut group (FW vs. F) was accompanied with potentially adverse body mass gain increase and hyperinsulinemia. Furthermore, aortic iNOS protein level increase as herein observed suggests development of local inflammation (Tejero et al. 2019). In addition, attenuated NCX1 may lead to slower efflux of Ca^{2+} from VSMC and its prolonged contraction. Observed walnut effects in aorta are thus by their nature fully consistent with finding of Scott and co-workers (Scott et al. 2017), showing that dietary walnuts were associated with modest favourable effects on blood pressure in wild type mice, but also with a combination of adverse and beneficial effects in mice with metabolic syndrome.

Surprisingly, in our study FRD alone (in absence of walnuts) increased pAkt level and decreased aortic LTCC alpha level, which can be assessed as vasodilatory and beneficial. In fact, increase in pAkt level could be due high insulin level in rats from FRD group. Since we used the antibody that recognises all Akt isoforms, this increase may also include Akt1 and indicate FRD-related VSMC proliferation (Jung et al. 2000).

In striking agreement with our findings regarding aortic KATP channel in rats from W vs. C group, the eicosapentaenoic acid (EPA) treatment significantly induced cardiac SUR2B mRNA expression, whereas Kir6.1 were unaffected by the EPA treatment (Tsuburaya et al. 2011). Levels of the EPA and ALA are significantly raised in blood by the intake of walnuts in humans (Marangoni et al. 2007), and dietary ALA is predominantly metabolised to EPA (Brenna et al. 2009).

Two-way ANOVA followed with post-hoc test did not confirm any significant effect of walnut diet on eNOS expression, in contrast to our expectations. Namely, walnuts contain considerable amounts of L-arginine, which is the precursor amino acid for NO biosynthesis (Xiao et al. 2018) and improves serum NO level (Joukar et al. 2017). The effects of LA and ALA, highly represented in walnut kernel, on endothelial cell functions and the related gene expression are influenced by LA/ALA ratios and their concentrations, and the mechanism may be partially mediated through NO/eNOS signalling pathways (Yang et al. 2018). On the other hand, eNOS-derived NO blocks Ca^{2+} influx through the sarcolemmal LTCC in cardiac myocytes (Farah et al. 2018). Moreover, there was a link among the levels of eNOS and arterial NCX1. They are associated in endothelial cell caveolae of mesenteric resistance arteries (Lillo et al. 2018). Concerning K^+ channels, it is established that NO activates soluble guanylyl-cyclase and K^+ channels. The NO also controls basal ERK1/2 phosphorylation by a signalling cascade that involves Kv1.2 (Palen et al. 2005). Despite previously reported associations of the studied proteins with eNOS/NO system, in the context of our results, we cannot link the observed changes in LTCC, NCX1 and KATP subunit SUR2B level with eNOS.

Walnuts are rich source of LA, while arachidonic acid is its metabolite (Croitoru et al. 2019). Our finding that walnut diet increased Akt phosphorylation is in concordance with the data showing that phosphorylation of Akt is enhanced in LA and arachidonic acid-treated HT29 and HepG2 cells (Mariniello et al. 2019). Hennig et al. (2006) suggests that both PI3K/Akt- and ERK1/2-mediated proinflammatory signalling events are critical in LA-induced endothelial cell activation and vascular inflammation. We did not observe increased activities of these pathways in ~~W~~FW vs. F group, but an increase of iNOS could be connected with high content of LA in walnut kernel (Croitoru et al. 2019). On the other hand, increased level of iNOS could be due to the observed increase of insulin concentration. Kumar et al. (Kumar et al. 2018) reported that hyperinsulinemia is accompanied with an increase in iNOS in epididymal white adipose tissue. In contrast, treatment with whole walnut extract prevented inflammation-induced upregulation of iNOS expression (Thangthaeng et al. 2018).

There is a growing evidence that in VSMC the Ca^{2+} influx during membrane depolarisation is regulated by the protein kinases. This regulation is presumably mediated by phosphorylation(s) of the L-type Ca^{2+} channel protein, or of an associated regulatory protein (Xiong and Sperelakis 1995). The AMPK can induce smooth muscle relaxation directly by a decrease of Ca^{2+} influx or of Ca^{2+} channel sensitivity. Findings of Schubart et al. demonstrate a new

role of AMPK in the control of actin cytoskeletal dynamics, potentially allowing long-term dilation of microvessels without substantial changes in cytosolic Ca^{2+} (Schubert et al. 2017). However, we did not observe any change in AMPK expression and phosphorylation which could be connected with walnut diet – related LTTC alterations.

Numerous studies regarding effects of walnut consumption on vascular function in humans are summarised recently by Morgillo et al. (2019). Vascular function was addressed measuring flow-mediated dilation, arterial stiffness, platelet activation, endothelium-dependent vasodilatation, vascular reactivity, total peripheral resistance, baroreflex sensitivity, circulating adhesion molecules etc. Morgillo and co-authors (2019) failed to find consistent results from walnuts, with only two of the chronic studies showing an improvement in vascular function. In contrast to the animal studies, humans are unsuitable for molecular study of vascular function, because vascular tissue samples are not easily available. Obtained results of the present study emphasising the role of LTCC and SUR2B expression as well as Akt phosphorylation in walnut effect accomplishment, could be useful in explanation of some effects of walnuts observed in humans. For example, the improvement in flow-mediated circulation in brachial artery of healthy persons whose diet was supplemented with walnuts (in amount equivalent to applied rat dose) observed in study of Bhardway et al. (2018), could be connected with mechanism suggested in this study. In contrast, insight in the vascular molecular events in rats make us more vigilant regarding favourable effects of walnuts in metabolic disorder, compare to Ma et al. (2010), which found walnut diet-related improvement of endothelium-dependent vasodilatation in brachial artery of Type 2 diabetes patients.

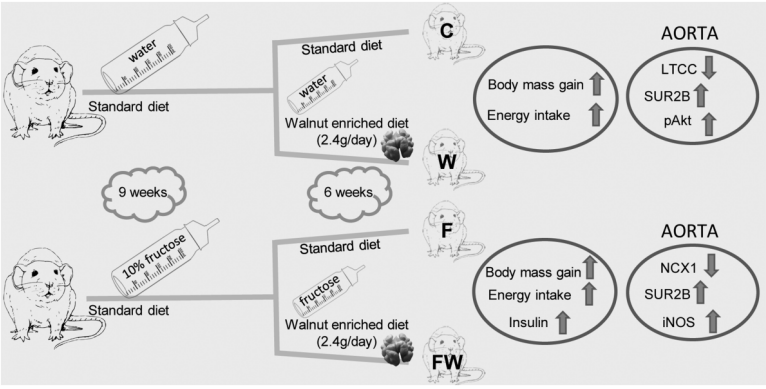
Strengths and weaknesses

This study is one of the rare molecular analyses of walnut effects on key proteins involved in vascular tone regulation in health and metabolic disorders. In addition, it is a real-life intervention study, because rats have consumed walnuts in a way and amount equivalent to those usual for humans. Regarding weakness, in this experimental setup it was not feasible to separate walnut effects on endothelial and vascular smooth muscle cells. Furthermore, to strengthen physiological impact, one direction for further research could be study on walnut effects on smaller arteria responsible for blood pressure regulation. Treatment of rats with extracted walnut compounds, such as individual fatty acids or polyphenols is also of interest to identify those responsible for the observed effects.

Conclusion

Results of our study seems to indicate that walnuts consumption can contribute to cardiovascular health, but in already developed metabolic syndrome milieu walnuts consumption also have some adverse effects (Figure 4). In conclusion, walnuts consumption is beneficial for cardiovascular health and should be incorporated in daily dietary regime before the onset of pathological process that results in vascular disturbances. Forward studies could be directed on walnuts' potential to prevent or minimise vascular damages that are due to different pathologies. Also, further experiments are needed to resolve the enigma of exact molecular mechanism of walnuts' vasculo-protective action.

Figure 4. Walnut effects on proteins involved in vascular tonus regulation in healthy rats and rats subjected to fructose overload. Abbreviations: C: control; W: walnut; F: fructose; LTCC: L-type Ca^{2+} channel; SUR2B: subunit of ATP-sensitive K^{+} channel; NCX1: Na^{+}/Ca^{2+} exchanger; pAkt: phosphorylated protein kinase B; iNOS: inducible nitric oxide synthase.



Disclosure statement

No potential conflict of interest was reported by the author(s).

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