Article

The Role of 20-HETE, COX, Thromboxane Receptors, and Blood Plasma Antioxidant Status in Vascular Relaxation of Copper-Nanoparticle-Fed WKY Rats

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Abstract: Recently, the addition of copper nanoparticles (NPs) in a daily diet (6.5 mg/kg) was studied in different animal models as a possible alternative to ionic forms. Male Wistar–Kyoto rats (24-week-old, $n = 11$) were fed with copper, either in the form of carbonate salt ($Cu_{6.5}$) or metal-based copper NPs ($NP_{6.5}$), for 8 weeks. The third group was fed with a half dose of each ($NP_{3.25} + Cu_{3.25}$). The thoracic aorta and blood plasma was studied. Supplementation with $NP_{6.5}$ decreased the Cu ($\times 0.7$), Cu/Zn-ratio ($\times 0.6$) and catalase (CAT, $\times 0.7$), and increased Zn ($\times 1.2$) and superoxide dismutase (SOD, $\times 1.4$). Meanwhile, $NP_{3.25} + Cu_{3.25}$ decreased the Cu/Zn-ratio ($\times 0.7$), and CAT ($\times 0.7$), and increased the daily feed intake ($\times 1.06$). Preincubation with either the selective cyclooxygenase (COX)-2 inhibitor, or the non-selective COX-1/2 inhibitor attenuated vasodilation of rat thoracic aorta in the $NP_{6.5}$ group exclusively. However, an increased vasodilator response was observed in the $NP_{6.5}$ and $NP_{3.25} + Cu_{3.25}$ group of rats after preincubation with an inhibitor of 20-hydroxyeicosatetraenoic acid (20-HETE) formation, and the thromboxane receptor (TP) antagonist. Significant differences were observed between the $NP_{6.5}$ and $NP_{3.25} + Cu_{3.25}$ groups of rats in: dietary intake, acetylcholine-induced vasodilation, and response to COX-inhibitors. Copper NPs in a standard daily dose had more significant effects on the mechanism(s) responsible for the utilization of reactive oxygen species in the blood plasma with the participation of prostanoids derived from COX-2 in the vascular relaxation. Dietary copper NPs in both doses modified vasodilation through the vasoconstrictor 20-HETE and the TP receptors.

Keywords: aging; 20-HETE; furegrelate; HET0016; indomethacin; NS-398; SQ-29,548; thromboxane-A_2

1. Introduction

Copper fluctuations in a diet may have either pro- or antioxidant effects on animal or human health, dependent on the daily dose [1–4]. A high intake of copper (including the recommended daily dose) may induce oxidation of lipids and proteins in cells that are potentiated in situations of a high-risk susceptibility to toxic compounds, such as in diabetes mellitus or hypertension [5,6]. Many of the major enzymes of biological processes are influenced by copper intake, including Cu–Zn superoxide dismutase (SOD),

cytochrome c oxidase, lysyl oxidase, L-ascorbate oxidase, monoamine oxidase, tyrosinase, and the enzymes of tryptophan degradation [7]. This may bring oxidative damage to lipids, proteins, and DNA, and result in neurodegenerative changes when dysregulated by either copper deficiency or its surplus [8,9].

Moreover, administration of copper nanoparticles (NPs) to animal feed, in a standard daily dose, may be of toxicological relevance due to its negative impact on animal health and the excretion of a large amount of this element into the environment and thus contamination [10]. Copper NPs induce a toxic effect by the increased production of free radicals, including hydroxyl radicals, hydrogen peroxides, and superoxide anions. The properties of metal NPs, including small size and high reactivity, increase their biological action, which may interfere with the physiological processes and the bioavailability of other macro- and microelements; therefore, the standard daily dose (6.5 mg/kg of diet) should probably be reduced to prevent increased toxicity [11].

As there are just a few studies regarding the safety of copper NPs in rats, and some of these results are controversial, we aimed to examine the influence of a standard 6.5 mg/kg dose of copper as NPs. In another group, the daily dose of NPs was reduced by half, to 3.25 mg/kg, and 3.25 mg/kg of copper carbonate was added instead. The third group was fed with 6.5 mg/kg of copper carbonate. The blood plasma antioxidant status was studied together with the participation of arachidonic acid metabolites in the vasodilator response of rat thoracic aorta to acetylcholine.

2. Materials and Methods

2.1. Drugs and Chemicals

Acetylcholine (chloride), indomethacin, noradrenaline (hydrochloride), and NS-398 were obtained from Sigma-Aldrich (St. Louise, MO, USA); copper as carbonate (purity $\geq 99\%$) from Poch (Gliwice, Poland); SQ-29,548, furegrelate, and HET0016 from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions (10 mM) of these drugs were prepared in distilled water, except for noradrenaline, which was dissolved in NaCl (0.9%) + ascorbic acid (0.01% *w/v*) solution; HET0016, SQ-29,548, and indomethacin were dissolved in ethanol; 1400 W in methanol; and NS-398 in DMSO. The solvent concentration was less than 0.01% (*v/v*).

These solutions were stored at $-20\,^\circ\text{C}$, and appropriate dilutions were made in Krebs–Henseleit solution (KH in mM: NaCl 115; $CaCl_2$ 2.5; KCl 4.6; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11.1) on the day of the experiment.

Metal-Based Copper Nanoparticles

Copper NPs (99.9% purity powder, 40–60 nm size, 12 m^2/g SSA, spherical morphology, 0.19 g/cm^3 bulk density, 8.9 g/cm^3 true density) were purchased from Sky Spring Nanomaterials (Inc., Houston, TX, USA). Stock solution (5 g/L) was prepared in a rapeseed oil, and about 9% of NPs dissolved as Cu (II) ions; thus, the final suspension contained both NPs and released copper species. The zeta potential of the copper NP suspension was determined to be -30.3 mV (in PBS) and -38.3 mV (pH 5), and the size was 104 nm (in rapeseed oil) determined by dynamic light scattering with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) [12].

2.2. Experimental Protocol

24-week-old normotensive Wistar–Kyoto (WKYs/NCrl) rats from Charles River (Sulzfeld, Germany) were allocated randomly to 3 groups (*n* = 11) and were fed individually for 8 weeks with experimental diets under standard laboratory conditions [1]. Exclusively male rats were studied, to enable comparison with the previous experimentations. The rats were fed with copper in a standard daily dose of 6.5 mg/kg, either as carbonate salt ($Cu_{6.5}$) or metal NPs ($NP_{6.5}$). Moreover, the third group ($NP_{3.25} + Cu_{3.25}$) was fed with 3.25 mg of copper NPs plus 3.25 mg of copper carbonate. Animal pellets were prepared according to the American Institute of Nutrition, and the copper NPs were dissolved in pure rapeseed oil (5 g/L) and mixed with the diet weekly [1].

2.3. Experimental Procedures

Intraperitoneal injections of ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) were used for anesthesia, followed by exsanguination [13]. Blood was centrifuged at $3000 \times g$ for 10 min to separate the plasma, which was further stored at $-80\ ^{\circ}\text{C}$ until analysis. The thoracic aorta was dissected and kept in an ice-cold Krebs–Henseleit buffer.

2.4. Blood Analysis

Copper and zinc were measured by the ICP–OES method. Bovine liver was used as a certified reference material (NIST1577C) for quality control. The units are expressed as μM. Superoxide dismutase activity was measured with Ransod and Ransel diagnostic kits (Randox); meanwhile, catalase (CAT) was determined by the enzymatic decomposition of hydrogen peroxide into water and oxygen. Data are expressed in U/mL. The sums of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined using an enzymatic method (Cell Biolabs) [14]. The units are expressed as μM. The total antioxidant potency FRAP (Ferric Reducing Antioxidant Power) was measured colorimetrically at 594 nm through the reduction of Fe(III) to Fe(II) by antioxidants present in the sample. Data are expressed as μM. The malondialdehyde (MDA) generates the MDA–TBA adduct, which was quantified with a fluorometric assay kit (ab118970) at Ex/Em = 532/553 nm. Data are expressed as mM.

2.5. Vascular Reactivity Studies

Briefly, aortic rings of 4 mm length were mounted in a stagnant 5 mL Graz Tissue Bath System (Barcelona, Spain) under the pre-load tension of 1 cN and aerated with carbogen gas for 60 min (TAM-A Hugo Sachs Elektronik, March, Germany) [15,16]. The functional integrity of aortic rings was checked with high K^+ (75 mM KCl) and ACh (10 μM). Next, aortic rings were incubated for 30 min with either the inducible nitric oxide synthase (iNOS) inhibitor (1 μM, 1400 W), the selective cyclooxygenase-2 (COX-2) inhibitor (10 μM, NS-398), the non-selective COX-1/2 inhibitor (10 μM, indomethacin), the inhibitor of 20-hydroxyeicosatetraenoic acid (20-HETE) formation (0.1 μM, HET0016), the thromboxane-A_2 synthase inhibitor (1 μM, furegrelate), or the thromboxane-A_2 receptor (TP) antagonist (1 μM, SQ-29,548), and contracted with noradrenaline (0.1 μM). Then, the cumulative doses of ACh (0.1 nM–10 μM) were added into the incubation chambers. Only one cumulative concentration–response curve (CCRC) was performed on each aortic ring.

2.6. Data Analysis and Statistics

Vascular relaxation was expressed as a percentage of the contractile response to noradrenaline NA (0.1 μM). The CCRCs were analyzed by a nonlinear regression model (log agonist vs. response), which determined the area under the curve (dAUC), the maximal response (E_{max}, %), and the potency ($pEC_{50} = -\log EC_{50}$). The Gaussian distribution of residuals and homoscedasticity of variance were tested for all data with $n = 11$; "n" refers to independent values, not replicates. The group comparison was performed by either a parametric (*t*-test or ANOVA) or non-parametric test (Mann–Whitney U-test or Kruskal–Wallis test). Results are expressed as the means $\pm$ SEM (for CCRCs) and means $\pm$ SD. Due to the small group sizes ($n < 12$), outliers detected by Grubbs' test were included in a data set [13]. The level of significance was when * $p < 0.05$.

3. Results

3.1. The General Characterization of WKY Rats

These results are presented in Figure 1A–D. Experimental supplementation with $NP_{6.5}$ neither changed the body weight (BW) gain ($\times 1.0$, Figure 1C), nor the dietary intake ($\times 1.02$, Figure 1D). In the $NP_{3.25} + Cu_{3.25}$ group of rats, BW gain was not changed in a significant way ($\times 1.4$, Figure 1C), contrary to the significant increase in daily feed intake ($\times 1.06$, $p = 0.0008$, Figure 1D). No significant difference was observed in the BW gain between

$NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$ supplemented rats ($\times 1.4$, Figure 1C), as opposed to the increased daily feed intake ($\times 1.04$, $p = 0.0462$, Figure 1D).
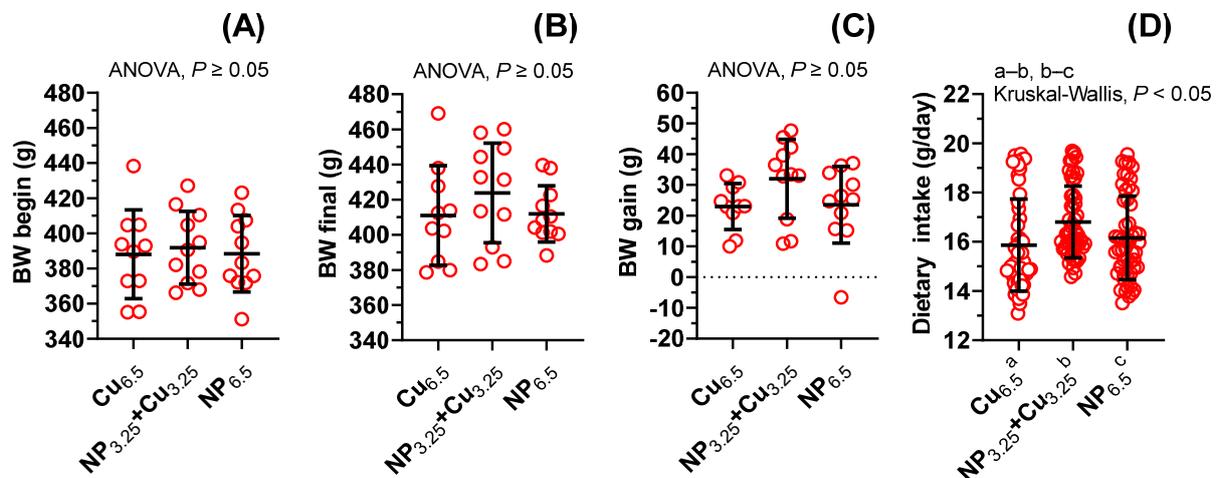

Figure 1. The influence of experimental diets on rat body weight (**A–C**), and daily feed intake (**D**). Values are expressed as means $\pm$ SD of $n = 11$ rats (**A–C**), and of $m = 56$ days of supplementation (**D**). $NP_{3.25} + Cu_{3.25}$ increased by 1.06-fold the daily feed intake.

3.2. Biomarkers of Oxidative Stress in the Blood Plasma

Supplementation with $NP_{6.5}$ modified the Cu/Zn-ratio ($\times 0.6$, $p = 0.007$), Cu ($\times 0.7$, $p = 0.0083$), CAT ($\times 0.7$, $p = 0.0134$), Zn ($\times 1.2$, $p = 0.0429$), and SOD ($\times 1.4$, $p = 0.0137$); meanwhile, $NP_{3.25} + Cu_{3.25}$ decreased the Cu/Zn-ratio ($\times 0.7$, $p = 0.0397$), and CAT ($\times 0.7$, $p = 0.0134$). $NP_{6.5}$ did not change FRAP ($\times 1.0$), GSH + GSSG ($\times 1.1$) and MDA ($\times 1.4$); and $NP_{3.25} + Cu_{3.25}$ did not change Cu ($\times 0.8$), MDA ($\times 1.0$), GSH + GSSG ($\times 1.0$), Zn ($\times 1.1$), FRAP ($\times 1.1$), and SOD ($\times 1.2$). There was no significant difference between $NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$ in the level of MDA ($\times 0.7$), GSH + GSSG ($\times 0.9$), SOD ($\times 0.9$), Zn ($\times 0.9$), CAT ($\times 1.0$), FRAP ($\times 1.1$), Cu ($\times 1.2$), and Cu/Zn-ratio ($\times 1.3$). Data are presented in Figure 2A–H.
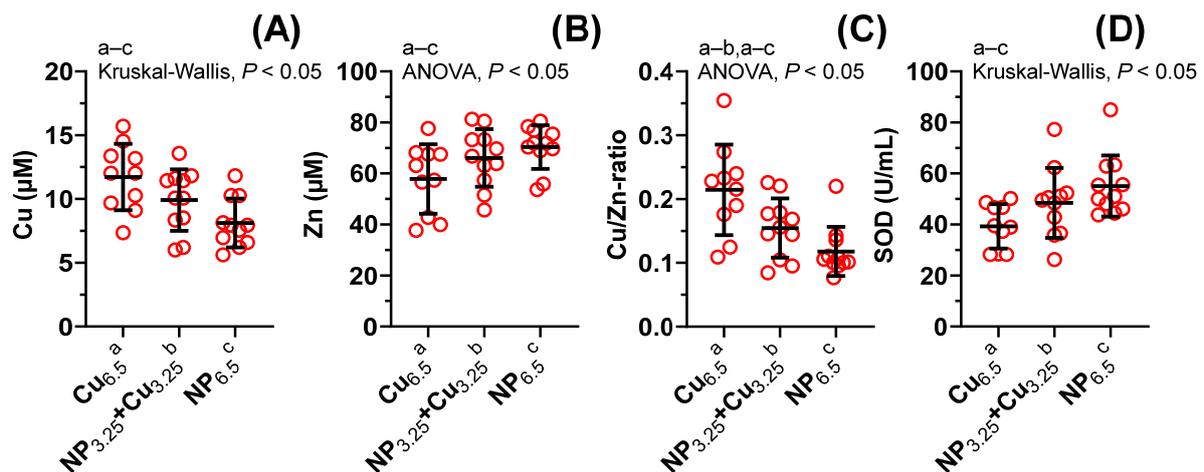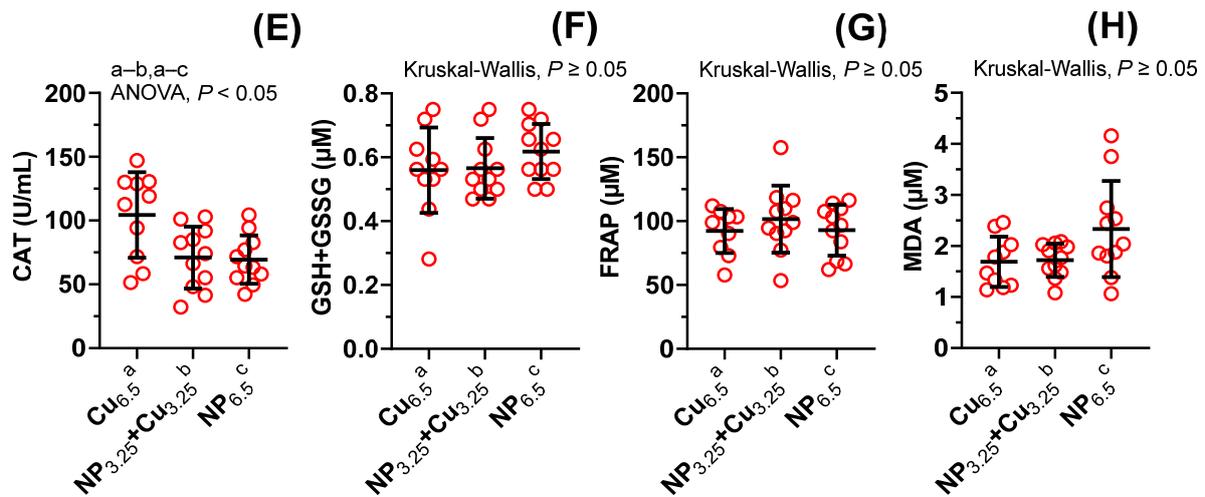

Figure 2. *Cont.*

Figure 2. The influence of experimental diets on Cu, Zn content (**A–C**), and antioxidant mechanism (**D–H**) in blood plasma. Values are expressed as means ± SD of $n = 11$ rats. Supplementation with $NP_{6.5}$ decreased the Cu (×0.7), Cu/Zn-ratio (×0.6), catalase (CAT, ×0.7), and increased Zn (×1.2), and superoxide dismutase (SOD, ×1.4). $NP_{3.25} + Cu_{3.25}$ decreased the Cu/Zn-ratio (×0.7), and CAT (×0.7).

3.3. Vascular Reactivity Studies

Neither $NP_{3.25} + Cu_{3.25}$ (AUC: ×0.85) nor $NP_{6.5}$ (AUC: ×1.18) changed the vasodilation compared to the control $Cu_{6.5}$. However, there was a tendency to increased vasodilation at 10 nM of ACh in $NP_{6.5}$ (see Figure 3). Moreover, significant change was observed in $NP_{3.25} + Cu_{3.25}$ compared to $NP_{6.5}$ (AUC: ×0.72). Preincubation with NS-398 diminished that response (between $NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$), which was completely abolished with indomethacin (see Table 1).
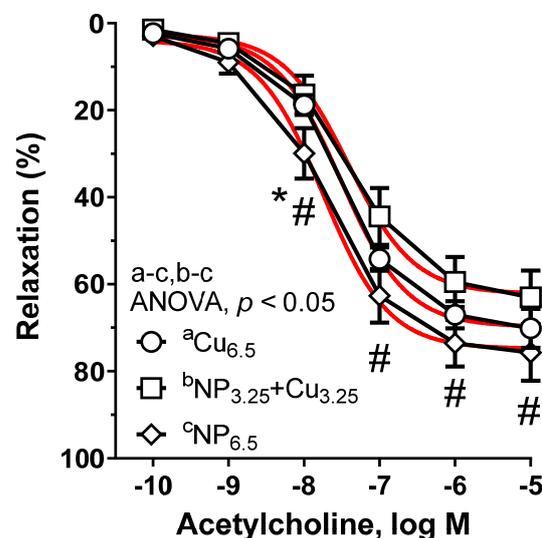

Figure 3. The relaxant response to acetylcholine in the isolated thoracic rings from rats supplemented with $Cu_{6.5}$, $NP_{3.25} + Cu_{3.25}$, and $NP_{6.5}$. Results are means ± SEM, * compared to $Cu_{6.5}$, [#] compared to $NP_{3.25} + Cu_{3.25}$, $p < 0.05$ of $n = 11$ rats; ANOVA/Tukey's. The red curve is the nonlinear regression model (log agonist vs. response). A significant change in the relaxant response was observed between the $NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$ groups of rats.

Table 1. The influence of iNOS inhibitor (1400 W, 1.0 µM), the selective COX-2 inhibitor (NS-398, 10 µM), the non-selective COX-1/2 inhibitor (indomethacin, 10 µM), the inhibitor of 20-HETE formation (HET0016, 0.1 µM), the thromboxane-A_2 synthase inhibitor (furegrelate, 1.0 µM), and the thromboxane receptor antagonist (SQ-29,548, 1.0 µM) on the vasorelaxant effects to acetylcholine of thoracic arteries from Wistar–Kyoto rats supplemented with $Cu_{6.5}$, $NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$.

Group	$Cu_{6.5}$				$NP_{3.25} + Cu_{3.25}$				$NP_{6.5}$		
	n	Emax (%)	pEC50	AUC	n	Emax (%)	pEC50	AUC	n	Emax (%)	pEC50	AUC
Control conditions	11	69.76	7.509	182.0	11	62.19	7.413	157.0	11	76.80 $	7.748	214.3 $
±SEM		1.899	0.087	12.73		3.460	0.172	25.01		3.294	0.147	25.10
+1400 W	5	74.95	7.581	205.9	5	66.40	7.454	173.2	5	80.15 $	7.839	232.1 $
±SEM		4.092	0.183	21.09		4.326	0.209	25.66		3.212	0.131	18.19
+HET0016	5	60.64	7.795	175.1	6	76.50 *#	7.386	190.3 *	7	88.44 *#$	7.874 $	252.9 *#$
±SEM		4.180	0.227	22.66		4.714	0.165	19.81		2.919	0.120	20.99
+SQ-29,548	5	75.77	7.210	176.2	7	82.08 *	7.222	199.0 *	6	86.82 *#	7.683 #$	241.4 *#
±SEM		3.725	0.139	17.27		2.855	0.102	15.66		2.929	0.112	17.28
+FURE	5	69.25	7.360	167.1	7	64.34	7.387	159.7	6	71.47	7.711	202.2
±SEM		2.888	0.124	12.77		3.030	0.143	16.53		4.186	0.195	27.60
+NS-398	5	73.82	7.582	207.5	5	61.61 #	7.277	152.7 #	5	70.36	7.624 $	190.9 *
±SEM		5.746	0.267	29.95		4.113	0.200	20.22		3.652	0.170	24.58
+INDO	5	76.61	7.564	206.6	5	67.88	7.618	182.8	5	68.88	7.540	179.8 *
±SEM		4.008	0.173	21.78		3.088	0.158	13.54		3.772	0.174	22.93

Values are based on the concentration–response curves shown in Figures 3–7. Data are expressed as means ± SEM where n represents the number of animals. * $p < 0.05$ compared with the control conditions, # $p < 0.05$ compared with the $Cu_{6.5}$ group, $ $p < 0.05$ compared with the $NP_{3.25} + Cu_{3.25}$ group as determined by one-way ANOVA followed by Tukey's post hoc test.
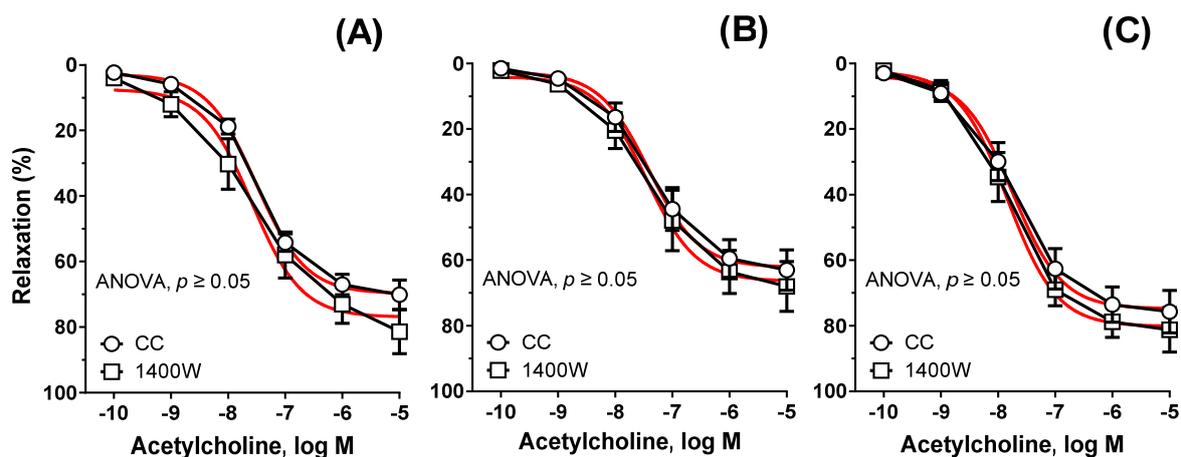

Figure 4. The influence of 1400 W on the relaxant response to acetylcholine. Aortic rings from rats supplemented with $Cu_{6.5}$ (**A**), $NP_{3.25} + Cu_{3.25}$ (**B**), and $NP_{6.5}$ (**C**) were pre-incubated with the inducible nitric oxide synthase inhibitor (1400 W, 30 min, 1 µM). Results are means ± SEM, $p > 0.05$ of $n = 5$ rats; two-way ANOVA/Sidak's. Preincubation with the selective iNOS inhibitor did not modify the vasodilation.

Preincubation with 1400 W (1 µM) did not modify the vasodilation in $Cu_{6.5}$ (AUC: ×1.13), $NP_{3.25} + Cu_{3.25}$ (AUC: ×1.04), and $NP_{6.5}$ (AUC: ×0.92), see Figure 4A–C.

Neither the selective COX-2 inhibitor (NS-398, 10 µM), nor the non-selective COX-1/2 inhibitor (indomethacin, 10 µM) changed the acetylcholine-induced response in the following groups of rats: $Cu_{6.5}$ (AUC: ×1.09, and ×1.10, respectively, Figure 5A), and $NP_{3.25} + Cu_{3.25}$ (AUC: ×0.90, and ×1.05, respectively, Figure 5B). However, a decreased response was observed in the $NP_{6.5}$ group (×0.84, and ×0.81, respectively, Figure 5C). There was no significant difference between NS-398- and indomethacin-induced response in $NP_{6.5}$ (AUC: ×1.04), opposite to some changes in $NP_{3.25} + Cu_{3.25}$ (AUC: ×0.86).

Preincubation with HET0016 (0.1 µM) potentiated vasodilation in $NP_{3.25} + Cu_{3.25}$ (AUC: ×1.39, Figure 6B), and $NP_{6.5}$ (AUC: ×1.52, Figure 6C) fed rats. This was not observed in the control group $Cu_{6.5}$ (AUC: ×1.02, Figure 6A).
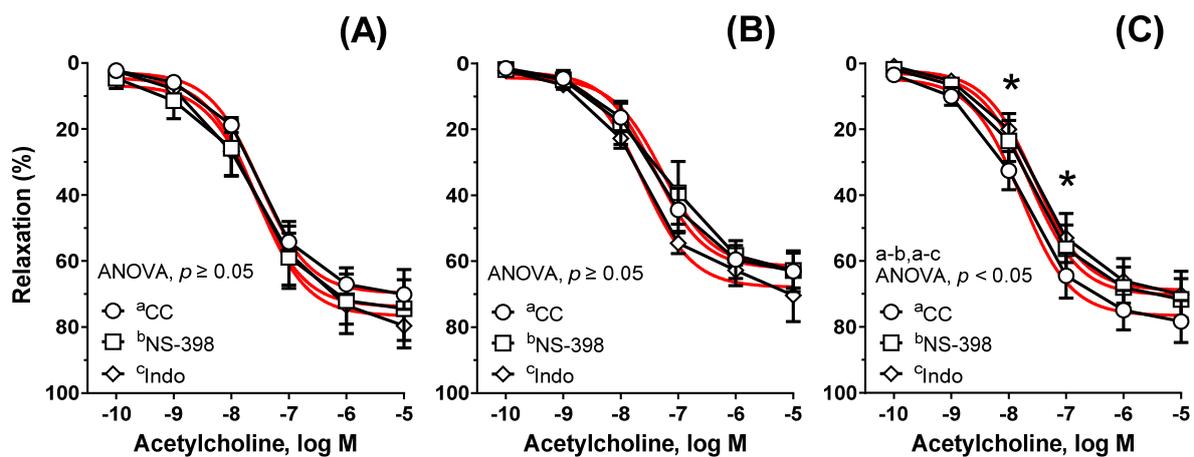
Figure 5. The influence of NS-398 and indomethacin (Indo) on the relaxant response to acetylcholine. Aortic rings from rats supplemented with $Cu_{6.5}$ (**A**), $NP_{3.25}$ + $Cu_{3.25}$ (**B**), and $NP_{6.5}$ (**C**) were pre-incubated with the selective cyclooxygenase-2 (COX-2) inhibitor (NS-398, 30 min, 10 μM) and the non-selective COX-1/2 inhibitor (Indo, 30 min, 10 μM). Results are means $\pm$ SEM, * $p < 0.05$ of $n = 5$ rats; ANOVA/Tukey's. Preincubation with NS-398 and indomethacin attenuated vasodilation of rat thoracic aorta in the $NP_{6.5}$ group exclusively.
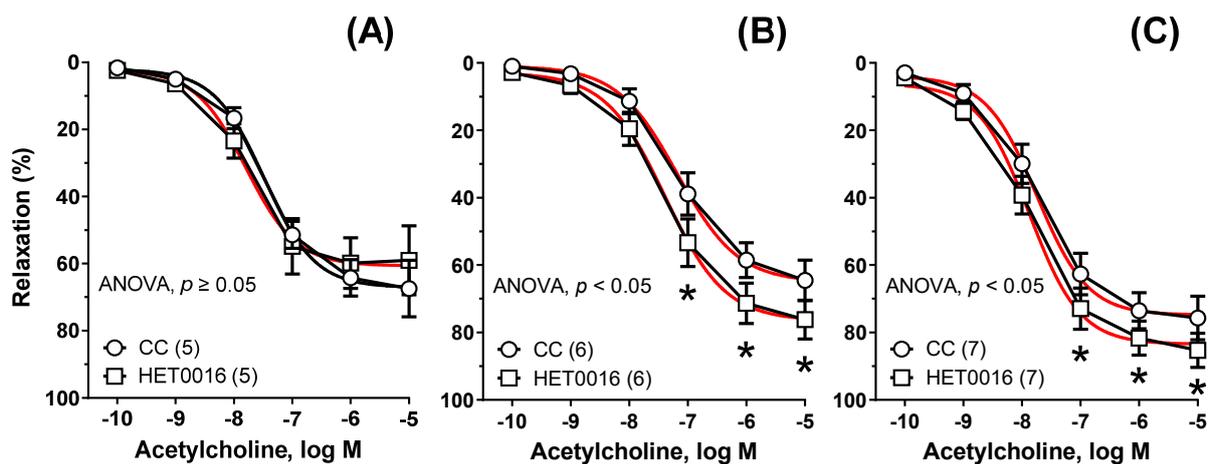

Figure 6. The influence of HET0016 on the relaxant response to acetylcholine. Aortic rings from rats supplemented with $Cu_{6.5}$ (**A**), $NP_{3.25}$ + $Cu_{3.25}$ (**B**), and $NP_{6.5}$ (**C**) were pre-incubated with an inhibitor of 20-HETE formation (HET0016, 30 min, 0.1 μM). Results are the means $\pm$ SEM, * $p < 0.05$ two-way ANOVA/Sidak's. Number of animals is indicated in parenthesis. Preincubation with HET0016 potentiated vasodilation in $NP_{3.25}$ + $Cu_{3.25}$, and $NP_{6.5}$ fed rats. This was not observed in the control group ($Cu_{6.5}$).

Neither the thromboxane-A_2 synthase inhibitor (furegrelate, 1 μM) nor the TP antagonist (SQ-29,548, 1 μM) changed the acetylcholine-induced response in $Cu_{6.5}$ (Figure 7A). However, in the $NP_{3.25}$ + $Cu_{3.25}$ (AUC: $\times 1.38$, Figure 7B) and the $NP_{6.5}$ (AUC: $\times 1.57$, Figure 7C) groups of rats, SQ-29,548 potentiated vasodilation. Furegrelate did not modify that response (AUC: $\times 1.05$, and $\times 1.03$, respectively). Moreover, a significant increase was also observed between SQ-29,548 and furegrelate (AUC: $\times 1.31$, and $\times 1.53$, respectively) (Figure 7B,C).

The vasodilator response to acetylcholine is presented in Figures 3–7. Results are expressed as E_{max} (%), pEC_{50} and AUC, see Table 1.
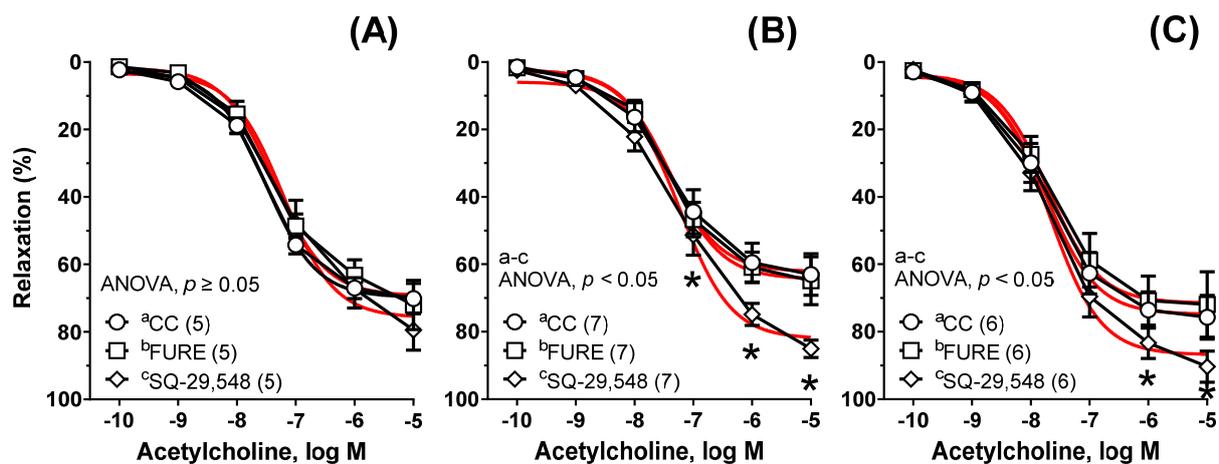
Figure 7. The influence of furegrelate and SQ-29,548 on the relaxant response to acetylcholine. Aortic rings from rats supplemented with $Cu_{6.5}$ (**A**), $NP_{3.25} + Cu_{3.25}$ (**B**), and $NP_{6.5}$ (**C**) were pre-incubated with the thromboxane-A_2 synthetase inhibitor (FURE, 30 min, 1 μM) and the thromboxane-A_2 receptor antagonist (SQ-29,548, 30 min, 1 μM). Results are means $\pm$ SEM, * $p < 0.05$; ANOVA/Tukey's. Number of animals is indicated in parenthesis. SQ-29,548 potentiated vasodilation in the $NP_{3.25} + Cu_{3.25}$ and in the $NP_{6.5}$ group of rats.

4. Discussion

Our previous studies revealed differences in the vascular tone regulation and the antioxidant status of rats supplemented with copper NPs (of 40–60 nm size) [1,2,12,17,18]. Of great importance is that in the previous experiments, both the age (either 4, 5, or 6 weeks) of Wistar Han IGS rats and the duration of feed intake (either 4 or 8 weeks) were what differentiated these studies from the one presented now, which can be described as 24 + 8 (24 weeks of age + 8 weeks of experimental feeding); and this was carried out on Wistar–Kyoto (WKY) rats, as a control for the spontaneously hypertensive rat (SHR) model which was also analyzed, but will be described elsewhere. Based on the previous results, antioxidant status and the participation of arachidonic acid metabolites were further investigated in the regulation of the vasodilator response induced by acetylcholine. Three different diets (i) standard with copper carbonate ($Cu_{6.5}$), (ii) with metal copper NPs ($NP_{6.5}$), and (iii) half dose of each ($NP_{3.25} + Cu_{3.25}$) were prepared in the form of pellets, and given daily to rats in order to study the physiological properties of dietary copper NPs.

Experimental supplementation with $NP_{6.5}$ neither modified the body weight nor the feed intake, which is in agreement with our previous results 4 + 8 (4 weeks of age + 8 weeks of experimental feeding), and 5 + 4 (5 weeks of age + 4 weeks of experimental feeding) [2,3,12,17]. However, in the $NP_{3.25} + Cu_{3.25}$ group of rats, we observed a significant increase in the daily feed intake compared to $Cu_{6.5}$ and $NP_{6.5}$, and an increase of body weight gain (not significant), which might be explained by the higher feed intake, and may become significant when more rats per group are studied. The observed increase in feed intake is difficult to explain and merits further investigation. Experimental treatment with $NP_{6.5}$ markedly reduced blood plasma Cu and increased Zn, which resulted in a decreased Cu/Zn-ratio. This is opposite to the 4 + 8 study, when Zn remained unchanged. However, in the same study, Cu and the Cu/Zn-ratio also decreased [18]. In the $NP_{3.25} + Cu_{3.25}$ group of rats, the Cu/Zn-ratio decreased significantly, which was due to a decrease in Cu and an increase in Zn (both results were not significant). There was no statistically significant difference between $NP_{3.25} + Cu_{3.25}$ and $NP_{6.5}$ in the Cu, Zn, and Cu/Zn-ratio. We have now observed, for the first time, increased activity of SOD and decreased CAT in $NP_{6.5}$ supplemented rats. This is contrary to our previous studies (4 + 8, and 6 + 8), when the activity of SOD was not modified and CAT increased [2,19]. However, another experiment from our research group (5 + 4) pointed to a decrease in CAT [12]. Increased SOD (result not significant) and decreased CAT were also observed for the $NP_{3.25} + Cu_{3.25}$ group. Increased SOD indicates an effective means of scavenging superoxide anion, whereas

decreased CAT points to possible enzyme depletion in response to the increased oxidative stress and intensified scavenging of hydrogen peroxide. Another enzyme of hydrogen peroxide degradation, glutathione peroxidase, was not modified in this study, and this is opposite to a significant decrease in the 5 + 4 study [12]. Copper in the form of NPs neither modified FRAP nor MDA. These findings are not entirely in agreement with our previous results (4 + 8 and 6 + 8), when FRAP increased [2,19], and MDA either increased (5 + 4 and 7 + 8) [1,12] or was not modified (4 + 8, and 6 + 8) [2,3,19]. We previously reported that replacing $Cu_{6.5}$ with $NP_{6.5}$ and reducing the dose (from a standard 6.5 mg/kg to a 3.25 mg/kg in the diet of rats 5 + 4) had particularly unfavorable effects on the respiratory system, causing adverse changes to the lungs. Surprisingly, these treatments also had a positive effect on the redox status of the liver and brain [20]. Moreover, the addition of copper NPs into the rat diet (5 + 4 and 7 + 8) reduced protein oxidation and nitration [1,12], as well as DNA oxidation and methylation. Meanwhile, lowering the daily dose increased the oxidation of proteins and DNA methylation [12]. In our study, neither $NP_{6.5}$ nor $NP_{3.25} + Cu_{3.25}$ modified the acetylcholine-induced vasodilation compared to $Cu_{6.5}$ control. However there was a tendency to increased vasodilation in $NP_{6.5}$ supplemented rats. This stays in agreement with the 4 + 8 study [2], and is in opposition to the 7 + 8 study, when $NP_{6.5}$ potentiated the vasodilator response induced by acetylcholine in a significant way [1]. Surprisingly, in the present study, $NP_{6.5}$ potentiated that response compared to $NP_{3.25} + Cu_{3.25}$. Preincubation with NS-398 (COX-2 inhibitor) diminished that response, which was completely abolished with indomethacin (COX-1/2 inhibitor). These results suggest participation of COX-2 in NP-induced response, which is a dose dependent mechanism. In another experiment, conducted by Cendrowska-Pinkosz et al. [21], $NP_{6.5}$ in the diet of rats did not change the acetylcholinesterase level (an enzyme that catalyzes the breakdown of acetylcholine) in the blood compared to $Cu_{6.5}$ (7 + 8), so the observed changes might not be due to enzyme depletion nor surplus. Even though we did not currently report any changes in acetylcholine-induced vasodilation followed by $NP_{6.5}$ and $NP_{3.25} + Cu_{3.25}$ intake (compared to the control $Cu_{6.5}$), the contribution of nitric oxide and arachidonic acid metabolites cannot be ruled out. We observed that iNOS inhibition with 1400 W did not modify that response in aortas from all three studied groups, which is contrary to the previous study (7 + 8), when the overproduction of NO from iNOS was engaged in vascular relaxation in the $NP_{6.5}$ group of rats [1]. Considering that the sensitivity of the arteries to nitric oxide was not altered (study with an exogenous NO donor sodium nitroprusside) [2], arachidonic acid derivatives may also be responsible for the vascular tone regulation of copper NPs, as we suggested previously [18]. Preincubation with either COX-2 or COX-1/2 inhibitors attenuated the vasodilator response in $NP_{6.5}$, indicating the involvement of a vasodilatory net effect of prostanoids origin from COX-2. This was neither observed for the control ($Cu_{6.5}$) nor when the dose was reduced by half (in the $NP_{3.25} + Cu_{3.25}$ group). The results with $NP_{6.5}$ are contrary to our previous report (4 + 8), which had revealed a decreased sensitivity of the smooth muscles to prostanoids (no significant change in acetylcholine-induced response after COX-1/2 inhibition) [2]. Given these results, the participation of another vasoconstrictor agent in vascular relaxation was studied. 20-Hydroxyeicosatetraenoic acid (20-HETE) is a potent metabolite of arachidonic acid. This reaction is mediated by cytochrome P450 and can be further metabolized by COX to 20-hydroxy compounds. Preincubation with a 20-HETE inhibitor potentiated the vascular relaxation in both $NP_{6.5}$ and $NP_{3.25} + Cu_{3.25}$ groups, indicating that 20-HETE is involved in acetylcholine-induced relaxation. However, this was not observed in young rats (7 + 8) [1]. Finally, we analyzed the contribution of the potent vasoconstrictor thromboxane-A_2 in the acetylcholine-induced response. However, the present results showed that thromboxane-A_2 is not an important vasoconstrictor candidate engaged in the vascular tone regulation of supplemented rats (studies with the thromboxane-A_2 synthesis inhibitor, furegrelate). Through the activation of thromboxane-A_2 receptors (TP), prostacyclin, prostaglandins, isoprostanes, and 20-HETE participate in the endothelial dysfunction associated with cardiovascular risk factors [15]. In this study, when the TP

were blocked with SQ-29,548, we observed increased vasodilation in both groups of copper NPs supplemented rats ($NP_{6.5}$ and $NP_{3.25}$ + $Cu_{3.25}$). These data point towards a potent vasoconstrictor that acts on the TP, suggesting 20-HETE as a possible candidate. However, other vasoconstrictors should also be taken under consideration in further studies [16]. As there is an interplay between vasoconstrictor and vasodilator factors, vasodilators should also be analyzed; this will be done in another study.

These data point to some significant changes induced by metal copper NPs, which are age-dependent and are observed between younger (12-week-old) and older (32-week-old) male WKY rats. Indeed, age-related changes in blood vessels elasticity/stiffness might be exacerbated by daily diet [22]. Many recent studies have focused on dietary intervention to improve vascular health and delay the onset of vascular aging [23]. Dietary intervention may improve not only the vascular/cardiovascular impairment, but also vascular cognitive impairment and dementia. However, so far, only certain vitamins (vitamin E, folate), multi-nutrient formulations, and unsaturated fatty acids have shown some initial promise [24].

5. Conclusions

Our findings have shown that increased oxidative stress accompanies copper NP intake, which further modulates vascular relaxation with the participation of 20-HETE, through the thromboxane-A_2 receptors. When a daily dose of copper NPs was decreased by half, the interplay between COX metabolites was also modified; however, the Cu/Zn-ratio and CAT remained unchanged compared to the higher dose. Further studies should concentrate on animals with metabolic disorders.

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