Alteration in flow (shear stress)-induced remodelling in rat resistance arteries with aging: improvement by a treatment with hydralazine

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Aims The link between aging and vascular diseases remains unclear, especially in resistance arteries. As a decreased vasodilator capacity of the endothelium is usually described in aging, we hypothesized that arteriolar remodelling in response to a chronic increase in blood flow might be altered. In addition, we tested the capacity of a vasodilator treatment with hydralazine to restore remodelling, as we have previously shown that hydralazine has a potent effect on the process.

Methods and results Mesenteric resistance arteries (350 μm diameter) from 3- and 24-month-old rats were exposed to high blood flow (HF) and normal blood flow (NF), for 2 weeks by sequential ligating second-order arteries in vivo. In HF arteries, diameter increased by 21% when intraluminal pressure was 100 mmHg, in association with a rise in superoxide production in young rats. On the other hand, both diameter and superoxide levels failed to increase in old rats. Hydralazine restored HF-induced remodelling in old rats in association with an increased superoxide production and a decreased superoxide dismutase (SOD) expression. The SOD-mimetic 4-hydroxy-2,2,6,6-tetramethyl piperidinoxyl (TEMPOL) prevented the effect of hydralazine on the arterial diameter. In old rats, hydralazine increased the arterial diameter in HF arteries without increasing eNOS expression. Furthermore, hydralazine also restored HF remodelling in eNOS knockout mice.

Conclusion Thus, flow remodelling in resistance arteries failed to occur in aging but it could be restored by hydralazine via a reactive oxygen species-dependent mechanism. These findings may have serious pathophysiological consequences in situations requiring flow-dependent remodelling such as ischaemic and metabolic diseases, more frequent in the elderly.

KEYWORDS
Microcirculation; Remodelling; Blood flow; Aging; Nitric oxide; Reactive oxygen species

1. Introduction
Age is a major risk factor for cardiovascular diseases. Age-related decline in cardiovascular functions is in part attributable to reduced cardiac output and heart rate. Aging is also associated with important changes in structure and function of large arteries, including diameter alteration, wall thickening, and stiffening, as well as endothelial dysfunction. With advancing age, pathological situations such as hypertension and ischaemic diseases associated with diabetes or not are more frequent, thereby disturbing the flow-dependent arterial remodelling. Tissues perfusion is under the control of resistance arteries, which in contrast with large arteries do not develop calcification in old subjects, but the vascular network density and organization change, and in some cases, become less efficient. In a pathological context, improving microvascular remodelling and, consequently, blood flow supply to organs are thus key issues.

An enhanced metabolism induces a chronic increase in blood flow in the microcirculation triggering outward hypertrophic remodelling, in order to normalize shear stress. This arterial wall remodelling requires the activation of the nitric oxide (NO) pathway. Nevertheless, in aging such an effect may not take place as endothelium (NO)-dependent dilation is severely blunted. Thus, we aimed first at validating the assumption that flow-induced remodelling of resistance was affected by aging.

Increasing microvascular flow and thereby improving tissues perfusion is a vital concern in diseases, such as diabetes and hypertension, inducing ischaemic disorders. Whereas large arteries undergo outright wall thickening and calcification during aging, structural and functional
changes occurring in resistance arteries are much less pronounced, suggesting that microvascular alterations related to aging may be reversible. In previous studies, hydralazine, a non-specific vasodilator, has been shown to improve regional circulations and particularly mesenteric blood flow. Thus, we hypothesized that microvascular remodelling in aged rats might be improved by hydralazine.

At the vascular level, advanced age is linked to greater oxidative stress. Reactive oxygen species (ROS) may have deleterious or beneficial effects. Indeed, they are involved in the initiation and progression of a variety of vascular diseases, and also play a key role in the vascular enlargement of the carotid artery in a model of arterio-venous fistula inducing a large increase in blood flow. Thus, we also investigated the role of ROS in flow-dependent remodelling of resistance arteries in old rats in which basal oxidative stress is increased.

We conducted a functional and a biochemical study using a model of chronic increase (60–70%) in blood flow in rat mesenteric resistance arteries. The first aim of our study was to evaluate the incidence of aging on microvascular flow-induced remodelling. Our second objective was to test the reversibility of a potential defect in remodelling using hydralazine and finally we tested the role of ROS in the process.

2. Methods

2.1 Arterial ligation in rat mesenteric arteries

Adult male young (age, 3 months, n = 14) and old (age, 24 months, n = 21) Wistar rats (Janvier, Le Genest Saint Isle, France) were anaesthetized (isoflurane) and blood flow in the mesenteric resistance arteries were modified, as previously described. Briefly, two first-order feed arteries were alternatively ligated near their bifurcation into second-order arteries. The non-ligated middle artery was thus exposed to high blood flow (HF) in order to irrigate the tissue corresponding to the ligated arteries. The other arteries had normal blood flow (NF) (Figure 1A). The gut was then replaced in the abdominal cavity and the abdomen was sutured in two layers with 4-0 silk. After surgery, rats were left in the animal facility.

After 14 days, rats were anaesthetized and arterial blood pressure was measured in the femoral artery. They then were sacrificed in a CO2 chamber. The mesentery was then quickly removed and placed in ice-cold physiological salt solution (PSS) of the following composition: 130, NaCl; 15, NaHCO3; 3.7, KCl; 1.2 KH 2PO4; 1.2, MgSO4; 11, glucose; 1.6, CaCl2; and 5, HEPES, pH 7.4. Vessels were then incubated in this buffer on ice for 30 min and then centrifuged (14,000 rpm, 15 min, 10 C). The detergent soluble supernatant fractions were retained, and protein concentrations in samples were equalized by using a Micro BCA Protein Assay Kit (Pierce). Proteins expression was measured in HF and NF arteries. Proteins concentration-response curves (1 nmol/L to 10 μmol/L) were then obtained after phenylephrine-induced preconstriction (50% of maximal contraction). Two CRC to acetylcholine were performed, before and after incubation with an inhibitor of NO synthase inhibitor, L-NAME (100 μmol/L, 20 min).

2.2 Animal treatment

Two groups of young and old rats were each divided into three groups (n = 7 per group):

- Group 1: young rats receiving tap water (control).
- Group 2: young rats receiving hydralazine (200 mg/L per day) in drinking water.
- Group 3: young rats receiving 4-hydroxy-2,2,6,6-tetramethyl piperidinioxy (TEMPOL, 20 mg/kg per day) by gavage.
- Group 4: old rats receiving tap water.
- Group 5: old rats receiving hydralazine (200 mg/L per day) in drinking water.
- Group 6: old rats receiving TEMPOL (20 mg/kg per day) by gavage-associated with hydralazine (200 mg/L per day) in drinking water.

Treatments started 24 h before surgery and were continued for 15 days. Hydralazine-supplemented water was changed every day.

2.3 Arterial diameter measurement in isolated arteries

Arterial segments were then cannulated at both ends in a video-monitored perfusion system (Living Systems Ins., Burlington, VT, USA) as described previously. Briefly, arteries were bathed and superfused with a Ca2+-free PSS containing EGTA (2 mmol/L) and sodium nitroprusside (10 μmol/L). Pressure was controlled by a servo-perfusion system and increased by steps from 10 to 150 mmHg. Internal diameter changes were measured and recorded continuously.

In a separate series of experiments, young mice (10–12 weeks) lacking the gene encoding for eNOS (−/−), treated or not with 200 mg/L hydralazine, and their littermate controls (+/−) (n = 4 per group) were submitted to arterial ligation. Arteries (HF and NF) were collected after 14 days as described above.

2.4 Histo-morphometric analysis

Arterial segments pressurized at 75 mmHg and fixed in a 4% buffered formaldehyde solution were cut using a cryostat. Transversal sections (7 μm thick) were stained with orcein solution. Then internal and external medial circumferences were assessed. From these values, medial cross-sectional area (CSA) was calculated, as described previously.

2.5 Vascular reactivity in isolated arteries

Other segments of mesenteric arteries were dissected and mounted on a wire-myograph (DMT, Aarhus, Denmark) as described previously. Briefly, two tungsten wires (25 μm diameter) were inserted in the lumen of the arteries and fixed to a force transducer and a micrometer, respectively. Arteries were bathed in a PSS as described above. Arteries were set to the baseline circumference L0 where L0 = 0.9L0100 is the internal circumference the artery would have in vivo when relaxed and under a transmural pressure of 100 mmHg. The near-maximal active wall tension of the vessel is developed at this circumference. Vessels were allowed to stabilize for 1 h. Artery viability was tested using a potassium-rich solution (80 mM).

Acetylcholine (ACh) and sodium nitroprusside (SNP) cumulative concentration–response curves (1 nmol/L to 10 μmol/L) were then obtained after phenylephrine-induced preconstriction (50% of maximal contraction). Two CRC to acetylcholine were performed, before and after incubation with an inhibitor of NO synthase inhibitor, L-NAME (100 μmol/L, 20 min).

2.6 Tissue extraction and protein expression

Segments of HF and NF arteries were collected and quickly frozen. Tissues were then pulverized in liquid nitrogen. The powders were resuspended in lysis buffer of the following composition: 10 mmol/L Tris–HCl, pH 7.4, 1% sodium dodecyl sulfate, 1 mmol/L sodium orthovanadate, and proteases inhibitors cocktail. Vessel extracts were incubated in this buffer on ice for 30 min and then centrifuged (14,000 rpm, 15 min, 10 C). The detergent soluble supernatant fractions were retained, and protein concentrations in samples were equalized by using a Micro BCA Protein Assay Kit (Pierce).

Proteins expression was measured in HF and NF arteries. Proteins (15 μg total protein from each sample) were separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with the primary antibody (1:1000 for eNOS from
Transduction Laboratories; 1:1000 for extracellular superoxide dismutase (ecSOD), 1:5000 for manganese SOD (MnSOD) and 1:1000 for copper/zinc SOD (Cu/ZnSOD) from Stressgen, washed (three times for 15 min), and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham) 90 min at room temperature. Proteins were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce Biotechnology). A polyclonal anti-actin antibody (Santa Cruz Biotechnology) was used to reprobe blots to confirm equal loading in lanes.

2.7 Superoxide detection and confocal microscopy

As previously described, dihydroethidium staining (DHE, Sigma-Aldrich) was used to evaluate the in situ levels of superoxide anions \( \text{O}_2^- \). DHE is permeable to cells and is oxidized by superoxide \( \text{O}_2^- \) to fluorescent products that are trapped by intercalation into the DNA. Sections were incubated with DHE (1 μmol/L) in phosphate-buffered solution (PBS) at 37 °C for 30 min in a humidified chamber protected from light. Fluorescent images of ethidium bromide were obtained using a confocal microscope (Solamere Technology, UT, USA). Sections incubated with PBS alone served as negative controls and sections from animals treated with lipopolysaccharides served as positive controls.

2.8 Statistical analysis

Results were expressed as means ± standard error (SEM). Significance of the differences between groups was determined by analysis of variance (ANOVA for repeated measurements for pressure–diameter curves and one-way ANOVA followed by Bonferroni for protein expression). In the other set of experiments, means were compared by unpaired t-test. P-values less than 0.05 were considered to be significant.

3. Results

3.1 Animals

Rat body weight was not significantly affected by hydralazine in young (308 ± 11 g in the presence of hydralazine,
3.2 Structural remodelling during aging and effect of hydralazine

Arteries submitted to high or normal blood flow were designated as HF and NF arteries, respectively.

3.2.1 Arterial diameter

In isolated mesenteric resistance arteries, stepwise increases in pressure induced a significant diameter enlargement (Figure 1).

In old control rats, passive arterial diameter, measured 14 days after arterial ligation, was similar in HF and in NF arteries (Figure 1C), whereas it was higher in HF than in NF arteries in young rats (Figure 1B).

In hydralazine-treated young (Figure 1B) and old rats (Figure 1C), passive arterial diameter was significantly higher in HF arteries than in NF arteries.

Hydralazine had no significant effect on arterial diameter in NF arteries in young (Figure 1B) or old rats (Figure 1C).

3.2.2 Cross-sectional area (Figure 1D)

In young and old control rats, medial CSA was significantly increased in HF arteries compared with that in the corresponding NF arteries.

In both NF and HF arteries, medial CSA was higher in old than in young rats, irrespective of the treatment.

In HF arteries, medial CSA was lower in hydralazine-treated rats (either young or old) than in non-treated rats.

3.3 NO pathway

3.3.1 Dilation to acetylcholine (Figure 2A)

Acetylcholine induced a significant dilation in mesenteric resistance arteries.

In both young and old control rats, the dilation induced by acetylcholine in HF arteries was equivalent to that in NF arteries.

In old rats, treated or not with hydralazine, acetylcholine-induced dilation was lower than in corresponding young rats, in either NF or HF.

NO synthesis blockade with L-NAME reduced acetylcholine-dependent dilation in all groups.

3.3.2 Dilation to sodium nitroprusside (Figure 2B)

Sodium nitroprusside induced a significant dilation in mesenteric resistance arteries, which was not affected by the chronic increase in blood flow, aging or hydralazine.

3.3.3 eNOS expression 14 days after ligation (Figure 3A)

The expression of eNOS was equivalent in all groups, irrespective of the type of artery (NF or HF), age or treatment.

3.3.4 Arterial remodelling in eNOS−/− mice arteries (Figure 3B)

In untreated eNOS−/− mice, passive arterial diameter in HF arteries was equivalent to that in NF arteries whereas in eNOS+/+ mice and in hydralazine-treated eNOS−/− mice, passive arterial diameter was higher in HF arteries than in NF arteries.

3.4 Reactive oxygen species

3.4.1 Superoxide detection

In young control rats, the level of superoxide was increased in HF compared with NF arteries, whereas in the old control rats, the level of superoxide was equivalent in NF and HF arteries.

In NF arteries, the level of superoxide was increased in old rats compared with young rats (Figure 4).

Moreover, in young rats treated with TEMPOL alone, the level of superoxide was equivalent in NF and HF arteries (1.07 ± 0.16 vs. 0.89 ± 0.38). The inhibition of superoxide level increase in HF arteries was associated with an absence of diameter enlargement (426 ± 26.3 μm in NF arteries vs. 450 ± 15.3 μm diameter in HF arteries when intraluminal pressure was 100 mmHg).

In HF arteries in old hydralazine-treated rats, the level of superoxide was increased compared with corresponding NF arteries and compared with HF arteries in old untreated...
rats. This increase in superoxide observed in HF arteries in old hydralazine-treated rats was abolished in old hydralazine + TEMPOL-treated rats (Figure 5A).

3.4.2 Arterial remodelling in old rats treated with hydralazine + TEMPOL (Figure 5B)
In old rats treated with hydralazine + TEMPOL, no significant remodelling was observed in HF arteries compared with NF arteries. Arterial diameter in NF arteries was not significantly affected by the combination of hydralazine and TEMPOL (Figure 5B compared to Figure 1C).

3.4.3 Superoxide dismutases expression (Figure 6)
In NF arteries, the expression of CuZnSOD, MnSOD, and ecSOD was increased in old control rats compared with the corresponding young group.

In HF arteries, the expression of CuZnSOD and ecSOD was decreased in hydralazine-treated old rats compared with control old rats (Figure 6B).
4. Discussion

We found that in old rats’ resistance arteries, a chronic increase in flow (shear stress) triggered a wall thickening but failed to induce luminal expansion. Nevertheless, in old rats treated with hydralazine high flow-induced remodelling was restored without change in endothelial function or eNOS expression but with an increased ROS production.

We used a model previously described in rats\textsuperscript{3,21,22} and mice,\textsuperscript{23} allowing comparison of resistance arteries exposed to different blood flow levels in the same physiological conditions \textit{in vivo}. In these arteries, chronic increases in blood flow induce outward hypertrophic arterial remodelling,\textsuperscript{3,23} allowing normalization of wall shear stress.

4.1 Impairment of microvascular flow-induced remodelling in old rats

A key result of the present study is that increasing blood flow in old rats failed to increase arterial diameter in resistance arteries whereas a wall thickening (hypertrophy) occurred.

Using a similar model, a previous report has shown that high flow-induced diameter enlargement in mature (8-month-old) was blunted compared with young rats (2-month-old).\textsuperscript{24} Our findings, obtained in rats three-time older, are in agreement with this study and with another work showing that high flow-induced remodelling was reduced in the uterine artery during pregnancy in 10-month-old compared with 3-month-old mice.\textsuperscript{25} In addition, we found that wall hypertrophy occurred in response to the chronic increase in blood flow in old rats, despite the absence of diameter enlargement. Thus, in aging the absence of increase in arterial diameter in response to high flow was associated with a further deleterious effect. Indeed, an increased intima and media thickness is directly associated with an increased risk of myocardial infarction and stroke in aged people.\textsuperscript{26}

In addition in old rats, we found reduced endothelial responsiveness to acetylcholine without, however, change in eNOS expression level. This is in accordance with previous reports showing that endothelium-dependent dilation is blunted in resistance coronary arteries with age.\textsuperscript{27,28} The influence of age on eNOS expression is more controversial and varies depending on the location of the studied arteries.\textsuperscript{29–31} The heterogenic role of NO is probably attributable to the variability of its interaction with the autonomic nervous system. Indeed the NO-dependent modulation of vascular tone diminishes with both aging\textsuperscript{32} and vessel size.\textsuperscript{33,34} However, two studies performed in resistance vasculature support our results, showing no influence of age on eNOS expression in rat mesenteric arteries.\textsuperscript{28,35} That endothelium-dependent dilation was affected without impairment in eNOS expression suggests that the activity of the protein may be reduced without change in expression level, as previously shown.\textsuperscript{36,37} This defect in NO bioavailability might explain the absence of remodelling in old rats. This is supported by the lack of arterial diameter enlargement in eNOS knockout.\textsuperscript{38}

In addition, a recent study has shown that in young rats, the remodelling following an increase in shear stress in the carotid artery depended on both NO and ROS production\textsuperscript{15} supporting the hypothesis of a mechanosensitive generation of ROS.\textsuperscript{39–41} In agreement with these findings obtained in conductance arteries, our results showed that a treatment with TEMPOL, a membrane-permeable SOD mimetic,\textsuperscript{42} abolished the diameter enlargement in association with an absence of increase in ROS in young rats. This suggests that microvascular remodelling depends on ROS production in young animals. In non-treated old rats, we observed an increased ROS production in NF arteries, in agreement with previous studies showing a rise in oxidative stress in aging.\textsuperscript{12,16} On the other hand, no rise in ROS was found in the HF arteries isolated from old rats. Thus, in old rats, the lack of increase in ROS, combined with the defect in eNOS-NO pathway, might emphasize the deficiency in flow-induced remodelling.

Figure 6 CuZnSOD, MnSOD, and ecSOD protein expression in mesenteric resistance arteries submitted to a chronic increase in blood flow (HF) compared with control arteries exposed to normal flow (NF). Arteries were isolated from old or young rats, treated or not with hydralazine (hydra). Protein expression is given as % of control. Values are means ± SEM (n = 7 per group). $^aP < 0.05$, old rats compared with young rats (within HF or NF groups). $^bP < 0.05$, effect of hydralazine (within HF or NF groups).
Our study was focused on the interaction between the endothelium and the smooth muscle in response to a chronic rise in blood flow. Although the present study did not take into account, the possible role of the autonomic nervous system in the remodelling, its role cannot be excluded. Indeed, its role is central in the control of vascular tone and further investigations are needed to elucidate its role in flow-remodelling.

4.2 Restoration of microvascular flow-remodelling by hydralazine in old rats

The second key finding of the present work is that hydralazine, an antihypertensive drug, restored high flow-induced remodelling in old rats in association with a reduction in wall mass. This represents a dual beneficial effect; an increase in diameter without hypertrophy. We also found in young rats that hydralazine reduced the hypertrophy associated with the diameter enlargement due to high flow, in agreement with our previous report.5

We used hydralazine, a well-known antihypertensive drug. Nevertheless, changes in pressure inducing remodelling per se,46 we chose a low dose not decreasing arterial blood pressure and thus not interfering with flow-induced remodelling. In this study, mean arterial pressure was effectively not significantly affected by hydralazine. Nevertheless, we cannot exclude that the decrease in blood pressure observed (6–7 mmHg) could however contribute to the recovery of luminal expansion by reducing vasoconstrictive influences and also take part in the decrease in wall thickness as previously shown.45 It was the unique drug to our knowledge that, when used at a low dose, afforded the double advantage of improving blood flow without significantly influencing arterial pressure.46 It is noteworthy that a high dose of hydralazine would have induced a decrease in both arterial pressure and blood flow.46

As detailed above, the high flow-induced remodelling depended both on NO production and on the production of ROS. Thus, we explored these two pathways in order to explain the restoration of high flow-remodelling in old rats treated with hydralazine.

In the present study, a chronic treatment with hydralazine improved the defective remodelling in mesenteric resistance arteries in old rats without increasing eNOS expression. Similarly, it has been shown that hydralazine does not affect eNOS protein expression and mRNA level in the kidney.47 In order to confirm that an enhancement in eNOS activity could not contribute to the restoration of remodelling, we treated eNOS-KO mice with hydralazine. Although eNOS-KO mice HF-arteries did not remodel (no diameter increase), a significant diameter enlargement occurred in hydralazine-treated eNOS-deficient mice. This latter remodelling was equivalent to that found in wild-type animals. This observation suggests that the restoration of flow-remodelling by hydralazine is independent of the eNOS-NO pathway. Nevertheless, we cannot dismiss the eventuality that the deficiency in flow-induced NO production, in the presence of hydralazine, could be compensated by an upregulation in INOS, nNOS or vasodilator prostaglandins production. Indeed, such an effect contributing to the maintenance of flow-induced dilation in non-treated eNOS-KO mice has been reported in coronary arteries49 and in gracilis muscle arterioles.50 Nevertheless, in the present study, the remodelling was totally abolished in eNOS-KO mice thereby excluding a role for these adaptive mechanisms in high flow-remodelling. In addition, in rats, hydralazine did not affect endothelium-dependent dilation, as previously shown in mice arteries.48 This also suggests that no compensatory mechanism was stimulated by hydralazine in old rats.

We found that in old rats treated with hydralazine, the recovery of flow-remodelling was associated with a higher superoxide anions level. This finding suggests that hydralazine improved flow-induced remodelling in old rats via a ROS-dependent mechanism.

In order to determine the origin of the increase in superoxide anions, we measured the expression levels of SOD, enzyme having superoxide-scavenging activity and whose alteration might induce an increase in superoxide anions level. We found that the expressions in cytoplasmic SOD (Cu/ZnSOD) and ecSOD, but not mitochondrial SOD (MnSOD), were reduced in this group. Therefore, the high level of superoxide observed in hydralazine-treated old rats HF arteries might result from a lower ROS scavenging by SOD. This is in agreement with a previous study showing that in the presence of hydralazine SOD is partially decomposed, thus inducing an increase in ROS concentration.51 Moreover, ROS generation was observed during oxidation of hydralazine.52 These observations might explain that hydralazine, usually described as antioxidant, can exert pro-oxidant effects as seen in the present study, in a situation involving high oxidative stress such as aged resistance arteries. Similarly, blood flow might also be responsible for oxidation of hydralazine, as increases in blood flow trigger vascular free radical generation.53 Therefore, the enhancement of superoxide production observed in HF arteries from hydralazine treated old rats could come from the combined influence of shear stress and aging.

Finally, that hydralazine induced a rise in ROS through SOD decomposition, resulting in a recovery of structural remodelling in old rats, was confirmed by the use of TEMPOL which totally suppressed the positive effect of hydralazine on HF-remodelling in old rats.

These observations suggest that $O_2^-$ take part in the improvement of flow-remodelling by hydralazine, strengthening the innovating hypothesis of a beneficial role of ROS in the process as previously described in carotid arteries,54 although the increase in superoxide anions found in the present study was attributable to a reduced SOD expression.

In conclusion, these findings show that, in resistance arteries, diameter enlargement induced by a chronic increase in blood flow was absent in old rats. Nevertheless, a chronic treatment with hydralazine restored this remodelling in old rats by increasing arterial diameter via an enhanced formation of reactive oxygen species. This defect in resistance arteries remodelling in response to a chronic increase in blood flow in aging should be taken into account in the understanding of the various diseases occurring more frequently in aging (cardiovascular, metabolic or ischaemic diseases). Nevertheless, that hydralazine was able to restore remodelling in old rats resistance arteries opens important perspectives as this finding shows that it remains possible to improve blood flow supply, often blunted with aging and the associated diseases.
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