Carrier-mediated transport controls hydroxyproline catabolism in heart mitochondria from spontaneously hypertensive rat

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Abstract In this study we have investigated hydroxyproline transport in rat heart mitochondria and, in particular, in heart left ventricle mitochondria isolated from both spontaneously hypertensive and Wistar-Kyoto rats. Hydroxyproline uptake by mitochondria, where its catabolism takes place, occurs via a carrier-mediated process as demonstrated by the occurrence of both saturation kinetics and the inhibition shown by phenylsuccinate and the thiol reagent mersalyl. In any case, hydroxyproline transport was found to limit the rate of mitochondrial hydroxyproline catabolism. A significant change in V_{max} and K_m values was found in mitochondria from hypertensive/hypertrophied rats in which the K_m value decreases and the V_{max} value increases with respect to normotensive rats, thus accounting for the increase of hydroxyproline metabolism due to its increased concentration in a hypertrophic/hypertensive state.

Key words: Hypertension; Hydroxyproline; Mitochondria; Transport; Collagen

1. Introduction

Among the amino acids present in mammalian tissue proteins, 4-hydroxyproline (HYP) exhibits a special feature: it is limited essentially to collagen and its biosynthesis and catabolism are therefore inseparably linked to these processes for collagen. In particular, HYP is released as a free compound from protease susceptible peptides and almost entirely catabolized. The major pathway of HYP catabolism in rat tissue is defined principally by enzymatic studies [1,2]: the first oxidized product of HYP is 3-OH-P5C, whose formation is catalyzed by mitochondria [2]; subsequently 3-OH-P5C is oxidized further to 4-OH-GLU, a substrate for a number of enzymes that act on glutamate, including transaminase [3], glutamine synthetase [4], glutaminase [5] and glutamate dehydrogenase [6]. At present, in spite of the mitochondrial localization of HYP catabolism, its transport across the mitochondrial matrix as well as the role of transport in HYP catabolism has not yet been exhaustively investigated. HYP transport has been studied in rat kidney mitochondria [7]; however, to date, no noteworthy information is available regarding HYP transport in rat heart mitochondria, even though the myocardium contains a collagen matrix that is a major determinant of its architecture, structural integrity and mechanical properties [8].

In this paper, we first show HYP carrier-mediated uptake in rat heart mitochondria, after which investigation is made of HYP transport in left ventricle mitochondria in a study carried out with spontaneously hypertensive rats and normotensive Wistar-Kyoto rats, both at 5 and 24 weeks of age. This is worthy of special attention since both the collagen concentration and the total amount of hydroxyproline content in the left ventricle were found to increase in spontaneously hypertensive rats as a result of increased fibroblastic activity in the pathogenesis of cardiac hypertrophy [8]. An increase in HYP translocator activity in hypertrophy/hypertension was shown. In all animals investigated, HYP transport across the mitochondrial membrane was found to limit the HYP oxidation rate.

2. Materials and methods

2.1. Chemicals

All reagents were of finest available grade with all solutions being adjusted to pH 7.4 by the addition of either Tris or HCl.

2.2. Animals

Two groups of male animals (Charles River) were used: spontaneously hypertensive rat and Wistar-Kyoto rats, with the latter used as a control. 5- and 24-week-old animals were considered for each group.

2.3. Arterial blood pressure and left ventricular mass index

Arterial blood pressure was measured by using the tail-cuff plethysmographic method (LE 5000 digital pressure meter, LETICA). The instrument functions by causing artery collapse (sphygmomanometer), utilizing pressure produced by a cuff; blood pulses are picked up by an appropriate transducer unit.

The values were taken at least three times with the mean value being reported.

2.4. Left ventricular hypertrophy

Left ventricular hypertrophy, determined by using the left ventricular mass index, was calculated based on the following formula: ventricle weight/body weight $\times 10^3$ [9–11].

2.5. Isolation of mitochondria

Spontaneously hypertensive and Wistar-Kyoto male rats weighing 150–350 g (5 and 24 weeks old, respectively) were anesthetized with ether, killed, and their hearts rapidly removed. 10 and 4 rats were used in a single preparation with 5- and 24-week-old rats, respectively. The left ventricle was isolated after trimming both atria, right ventricle and connective tissue.

Mitochondria from either rat whole heart or rat heart left ventricle were isolated essentially according to [12], using a medium consisting of 0.25 M sucrose, 20 mM Tris-HCl pH 7.25, 1 mM EGTA. The final mitochondrial pellet was suspended in the isolation medium to obtain

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Abbreviations: HYP, hydroxyproline; 3-OH-P5C, 3-hydroxypyrroline-5-carboxylate; 4-OH-GLU, 4-hydroxyglutamate

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30–40 mg protein/ml with mitochondrial protein measured according to [13].

Since coupled mitochondria are strictly required to investigate properly oxidative phosphorylation, control was performed, via the respiratory chain, in each experiment of the mitochondrial integrity and effectiveness in electron flow. In order to do so, oxygen uptake measurements were carried out at 25°C in 2 ml of a medium consisting of 100 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium phosphate buffer, 10 mM Tris-HCl pH 7.4, by means of a Gilson 5\6 oxygraph using a Clark electrode. Our mitochondrial preparations showed a respiratory control ratio, i.e. the ratio between the rate of O_2 consumption in state 3 (in the presence of ADP) and the rate in state 4 (in the absence of ADP), in the range 3–5. Moreover *P/O* ratios, i.e. the mole equivalents of phosphate esterified per mole oxygen consumed, were 3.0 ± 0.3, 2.3 ± 0.8 and 1.2 ± 0.2 as obtained by using glutamate plus malate, succinate and ascorbate plus TMPD as respiratory substrates, respectively.

2.6. Fluorimetric assay

Changes in the redox state of the pyridine nucleotide were followed fluorimetrically using a Perkin-Elmer luminometer LS-5 with excitation and emission wavelengths set at 334 and 456 nm, respectively. HYP uptake was monitored, according to [7], following intramitochondrial $NAD(P)^+$ reduction caused by externally adding the substrate to mitochondria previously incubated with 1.25 μM FCCP, to increase the intramitochondrial $NAD(P)^+$ concentration, and supplemented, 2 min later, with 2 μg rotenone, to prevent oxidation of the newly synthesized NAD(P)H via the respiratory chain. The rate of fluorescence change was measured as the tangent to the initial part of the experimental curve and expressed as nmol intramitochondrial $NAD(P)^+$ reduced/min per mg mitochondrial protein. In order to obtain a quantitative measurement of the rate of intramitochondrial $NAD(P)^+$ reduction, the fluorimetric response was calibrated as in [14].

2.7. Statistical analysis and computing

Results are expressed as mean values ± S.E.M. Statistical significance was determined by Student's *t*-test. Where necessary, experimental plots were obtained by means of Grafit software (Erithacus).

3. Results

3.1. Spectroscopic study of hydroxyproline transport in rat whole heart mitochondria

As expected in the light of mitochondrial localization of HYP-catabolizing enzymes ([2,7]; Fig. 1), the addition of HYP to rat heart mitochondria, preincubated with the uncoupler FCCP and then supplemented with rotenone, causes a rapid increase in fluorescence of the intramitochondrial pyridine nucleotides (not shown). In fact, HYP in the mitochondrial matrix is oxidized to 4-OH-GLU via HYP oxidase and 3-OH-P5C dehydrogenase with simultaneous reduction of the intramitochondrial $NAD(P)^+$ (Fig. 1). In order to gain an initial insight into the occurrence of carrier-mediated HYP transport, in the same experiment we also examined the ability of mersalyl and phenylsuccinate, used as impermeable inhibitors of certain mitochondrial carriers [15–18], to affect the rate of fluorescence increase. Both mersalyl (250 μM) and phenylsuccinate (5 mM) significantly decreased (about 55 and 40%, respectively) the rate of intramitochondrial NAD(P)H formation (not shown), thus suggesting that HYP transport is a carrier-mediated process.

On the basis of these findings, the question arises as to which step in HYP metabolism can limit the rate of NAD(P)H formation in vitro and likely in vivo. Indeed, since this could reflect either the rate of HYP uptake or the rate of one of the intramitochondrial reactions, investigation was made to determine the rate-limiting step in mitochondrial HYP catabolism. We found that Triton experiments failed

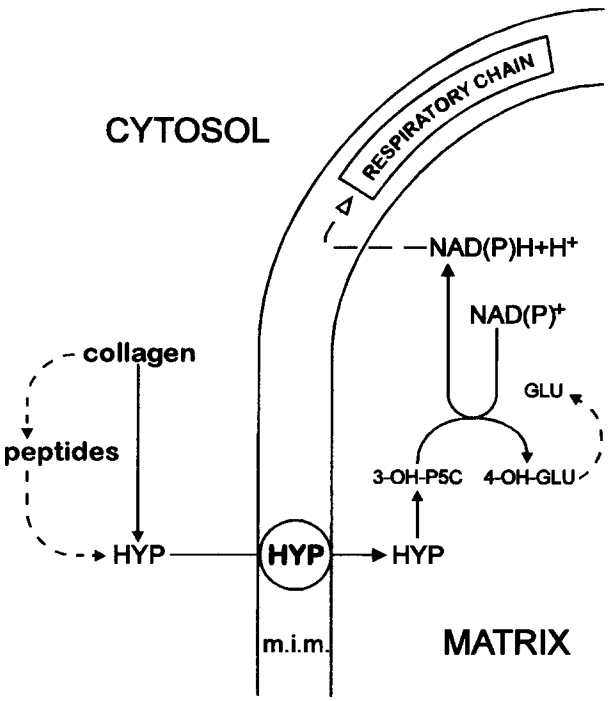

Fig. 1. Mechanism of mitochondrial HYP transport.

to elucidate this point: in fact, as in [7,17,18], externally added Triton completely prevents any change of fluorescence (not shown). Accordingly, as advised (see [7,17–20]), control strength criteria were applied by using the inhibitor phenylsuccinate, which can inhibit HYP transport but not catabolism, since it is a non-penetrant compound [15]. Thus, the dependence of the rate of fluorescence increase due to externally added HYP in either the absence or presence of increasing phenylsuccinate concentrations was studied as a Dixon plot (Fig. 2A). Phenylsuccinate inhibits the rate of fluorescence increase caused by externally HYP in a competitive manner ($K_i = 2.5$ mM). It should be noted that if the reciprocal of the rate of investigated reactions is plotted against inhibitor concentrations, the resulting Dixon plot extrapolated to zero inhibition provides a measure of the inhibited process, i.e. the transport (see [7,17–20]). In Fig. 2A, the intercept to the ordinate axis of the line given by linear regression analysis of the experimental points obtained in the presence of the inhibitor coincides with the experimental point obtained in the absence of phenylsuccinate; this shows that the measured rate of NAD(P) reduction reflects the rate of HYP transport across the mitochondrial membrane, i.e. the rate of HYP catabolism depends on HYP transport across the mitochondrial membrane, under these experimental conditions.

In another set of experiments, the dependence of the HYP uptake rate versus HYP concentration was investigated by means of a double-reciprocal plot (by using Grafit software) (Fig. 2B). HYP uptake reveals hyperbolic saturation characteristics: the K_m, i.e. the HYP concentration which gives half-maximum rate of NAD(P)H formation, and V_{max} values were 1.3 mM and 6 nmol/min per mg protein.

3.2. Arterial blood pressure and left ventricular mass index

In order to check metabolic alterations in both the initial and mature hypertension states, 5- and 24-week-old rats were

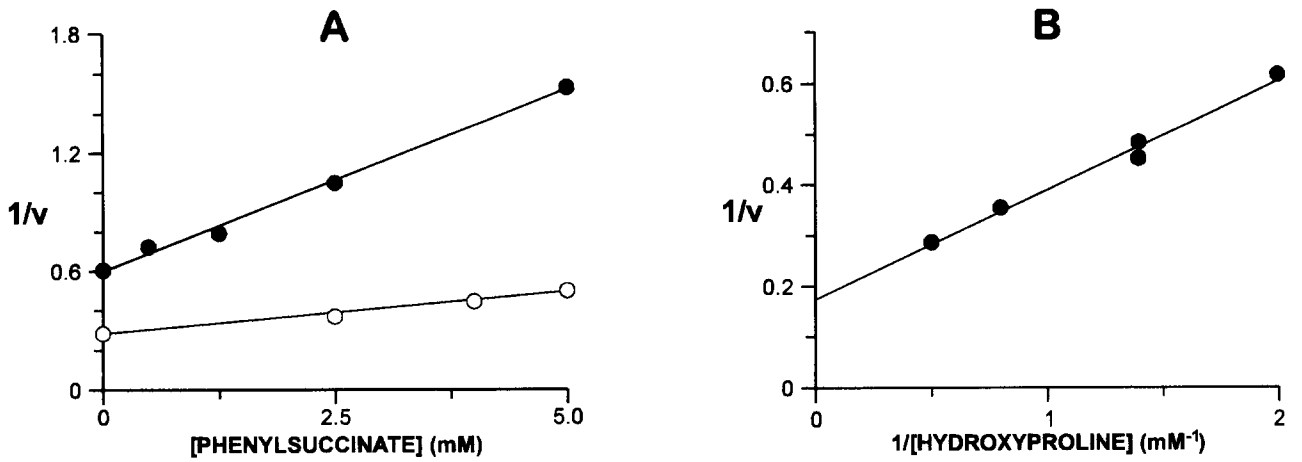

Fig. 2. Fluorimetric investigation of HYP uptake by rat heart mitochondria. Mitochondria (0.8 mg protein) from rat whole heart were preincubated at 20°C for 1 min in 2.0 ml of incubation medium consisting of 0.2 M sucrose, 10 mM KCl, 20 mM HEPES-Tris pH 7.2, 1 mM $MgCl_2$ in the presence of 1.25 μM FCCP and 2 μg rotenone (for details see Section 2). The HYP uptake reaction was started with hydroxyproline addition. (A) Dixon plot of the inhibition by phenylsuccinate. Phenylsuccinate (5 mM) was added 1 min before the addition of substrate. The reduction rate of intramitochondrial $NAD(P)^+$ was measured by using 0.7 mM (closed symbols) and 2 mM (open symbols) HYP in the absence or presence of phenylsuccinate at the indicated concentrations. (B) Kinetic analysis of HYP uptake. HYP was added at the indicated concentrations. The rate measured as the tangent to the initial part of the progress curve is expressed as nmol $NAD(P)^+$ reduced/min per mg mitochondrial protein.

used, with their arterial blood pressure and left ventricular mass index tested first. No significant difference in arterial blood pressure was found when comparing 5-week-old spontaneously hypertensive and Wistar-Kyoto rats (123 ± 10 and 115 ± 9 mmHg, respectively) whereas, in agreement with [11,21–23], the systolic blood pressure values were significantly different in spontaneously hypertensive and Wistar-Kyoto rats at the 24th week of life (spontaneously hypertensive rats, 214 ± 14 mmHg; Wistar-Kyoto rats, 139 ± 12 mmHg; $p < 0.001$).

A progressive increase in left ventricular mass index was measured with age in spontaneously hypertensive rats when compared to Wistar-Kyoto rats: the significant difference reported at the 5th week of age (spontaneously hypertensive rats, 3.1 ± 0.3; Wistar-Kyoto rats, 2.5 ± 0.3; $p < 0.05$), in agreement with [9,23,11], became consistent and more significant at the 24th week (spontaneously hypertensive rats, 3.4 ± 0.3; Wistar-Kyoto rats, 2.2 ± 0.3; $p < 0.001$). Interestingly, left ventricular mass indexes were found to be statistically different in 5- and 24-week-old spontaneously hypertensive rats ($p < 0.03$).

Thus, as no significant change in blood pressure was found in 5-week-old spontaneously hypertensive rats, this study assumes that they represent a model in which any biochemical change might be related to ventricle hypertrophy (hypertrophic state). In contrast, since in 24-week-old spontaneously hypertensive rats biochemical alterations should be considered in the light of the increase of both blood pressure and ventricular mass, they are assumed to be the result of a hypertrophic/hypertensive state.

3.3. *Spectroscopic study of HYP uptake into spontaneously hypertensive rat heart left ventricle mitochondria*

As HYP concentration and metabolism increase strongly in the hypertensive/hypertrophic states, comparison was made of heart left ventricle mitochondria from spontaneously hypertensive rats and normotensive strain 'control', in terms of their ability to internalize HYP. In this case, rat heart left ventricle mitochondria were used from both 5- (hypertrophic state) and 24-week-old (hypertrophic/hypertensive state) animals. As shown in Fig. 3, when using 24-week-old rats (C,D) the rate of fluorescence increase caused by externally added HYP (1 mM) was found to increase in spontaneously hypertensive rats (350%) compared to that found when using age-matched Wistar-Kyoto rats, whereas an approximately equal rate value was found when animals at the fifth week of age were used (A,B).

The HYP uptake induced fluorescence change appears to follow a first-order type reaction as calculated by plotting $2.3 \cdot \log(F_{HYP})_{MAX}/([F_{HYP}]_{MAX} - [F_{HYP}])$, where $(F_{HYP})_{MAX}$ = maximum fluorescence change, i.e. HYP accumulated, measured after equilibrium is reached, versus the indicated times t: the value of the first-order constant is about 0.6 min^{-1} for both normotensive rats at 5 and 24 weeks and spontaneously

Table 1
Kinetic parameters of HYP transport in both 5- and 24-week-old spontaneously hypertensive or Wistar-Kyoto rat heart left ventricle mitochondria

HYP translocator	SH_5	WKY_5	SH_{24}	WKY_{24}
K_m (mM)	1.3 ± 0.15	1.2 ± 0.09	0.4 ± 0.06	1.1 ± 0.12
V_{max} (nmol/min per mg)	6.3 ± 0.20	5.2 ± 0.12	12.7 ± 0.27	5.5 ± 0.16

Experimental conditions for the measurement of activity of HYP carrier in mitochondria from either 5- and 24-week old spontaneously hypertensive (SH) and Wistar-Kyoto (WKY) rats were as described in Section 2 and Fig. 2.
K_m and V_{max} values were calculated from a double-reciprocal plot obtained with three independent determinations of the rate of transport. Values are reported as means ± standard error as calculated by Grafit software.

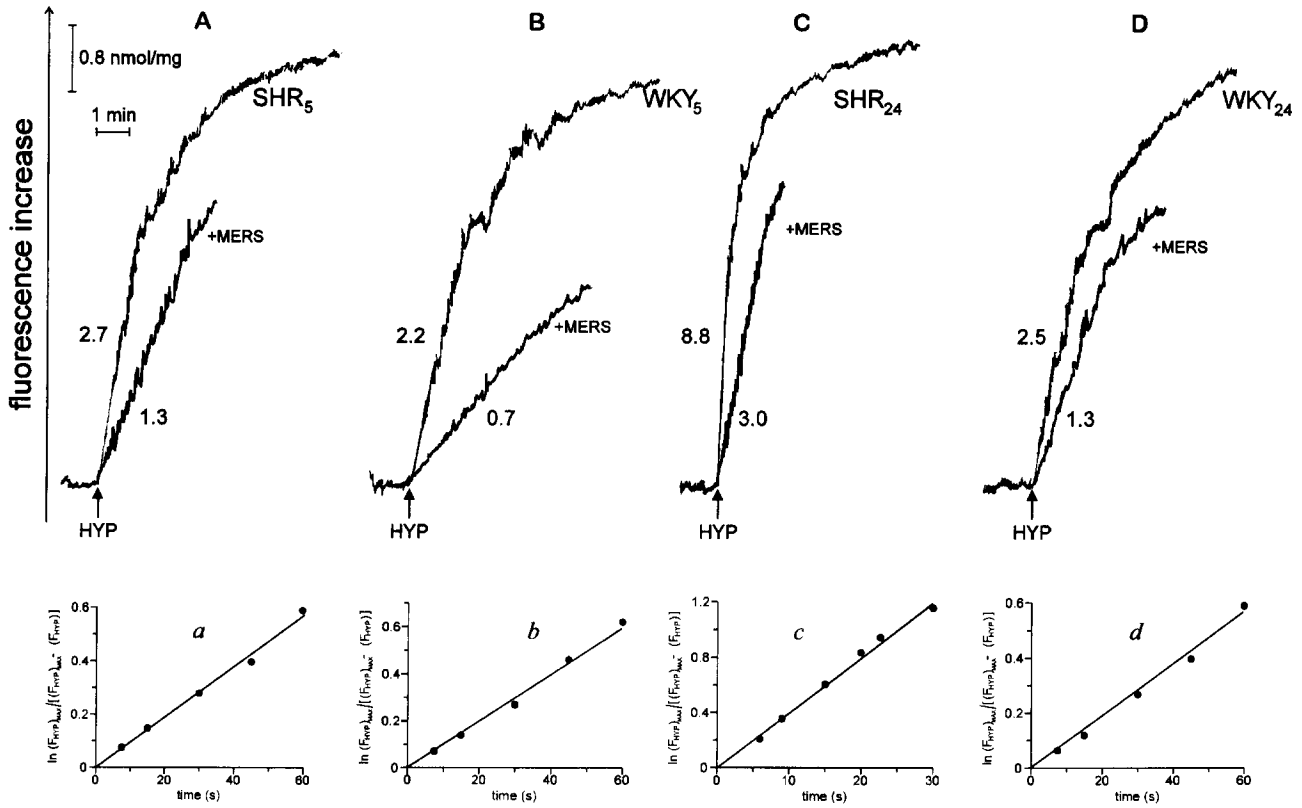

Fig. 3. Hydroxyproline uptake by rat heart left ventricle mitochondria from either 5- and 24-week-old spontaneously hypertensive or Wistar-Kyoto rats. Either 5- and 24-week-old spontaneously hypertensive rat heart left ventricle mitochondria (A,C) or 5- and 24-week-old Wistar-Kyoto rat heart left ventricle mitochondria (B,D) (each 0.6 mg protein) were suspended at 20°C for 1 min in 2.0 ml of incubation medium in the presence of 1.25 μM FCCP and 2 μg rotenone (experimental conditions as in Fig. 2). Where indicated, 1 mM hydroxyproline (1 mM, HYP) was added. Mersalyl (250 μM, MERS) was added 1 min before substrate addition. The numbers along the curves represent the rate measured as the tangent to the initial part of the progress curve, expressed as nmol $NAD(P)^+$ reduced/min per mg mitochondrial protein. Inset: logarithmic plot of HYP uptake induced fluorescence change value (F_{HYP}), demonstrating first-order kinetics: $2.3 \cdot \log(F_{HYP})_{MAX}/[(F_{HYP})_{MAX}-(F_{HYP})] = kt$, where $(F_{HYP})_{MAX}$ = maximum fluorescence change measured, i.e. HYP accumulated, after equilibrium is reached.

hypertensive mitochondria from 5-week-old animals, i.e. in the hypertrophic state (see insets a,b,d of Fig. 3), and 2.4 min^{-1} for spontaneously hypertensive rats at 24 weeks of age, i.e. in a hypertrophic/hypertensive state (see inset c of Fig. 3). In any case, mersalyl inhibits the process (Fig. 3); the nature of inhibition is non-competitive as determined from Dixon plots (Fig. 4). The K_i values for mersalyl were 500 and 280 μM for spontaneously hypertensive and 500 and 450 μM for Wistar-Kyoto mitochondria from 5- and 24-week-old rats, respectively. This shows that HYP carrier contains -SH group(s) located far from the substrate binding site. It should be noted that the mersalyl experiment provides evidence that HYP oxidation reflects the rate of the transport (see above) in both spontaneously hypertensive and Wistar-Kyoto rats at 5 and 24 weeks of age. The differences in K_i values, which reflect inhibitor affinity for the carrier, initially suggest that the carrier molecule is significantly modified in the hypertrophic/hypertensive state. When investigation of the dependence of the HYP transport rate on externally added HYP was carried out, saturation kinetics were found in all cases. In Table 1 comparison is made of mitochondria from spontaneously hypertensive and Wistar-Kyoto rats, both at 5 and 24 weeks of age, with respect to the kinetic parameters. The p values obtained by statistically evaluating the measurements of 3 experiments confirm that significant differences in both K_m and V_{max} values are found between the 24-week-old spontaneously hypertensive and the other mitochondria, as described in Table 1 ($p < 0.05$). By assuming the HYP cytosolic concentration to be about 1 mM (see [24–26]), actual rates of 2.8 and 9.3 nmol taken up HYP/min per mg mitochondrial protein for spontaneously hypertensive rats can be calculated in the hypertrophic and hypertrophic/hypertensive state, respectively (in age-matched Wistar-Kyoto rat heart left ventricle mitochondria the rates are 2 and 2.7 nmol/min per mg protein, respectively).

4. Discussion

The results reported in this paper lead to the conclusions that both HYP uptake into rat whole heart mitochondria occurs via a carrier-mediated process and that the rat heart left ventricle mitochondria tested differ from each other (spontaneously hypertensive with respect to Wistar-Kyoto rats) as far as hypertrophy/hypertension is concerned. In particular, we observed an increase in HYP catabolism due to the increase in HYP carrier activity which allows for HYP uptake by mitochondria where its catabolism takes place. At present, the reason for the increased V_{max} and decreased K_m values still needs to be established. The carrier activity increase observed at the 24th week of life in spontaneously hypertensive rats is consistent with increased collagen concentration which requires an increase in catabolism [27,28]; thus, the increase in

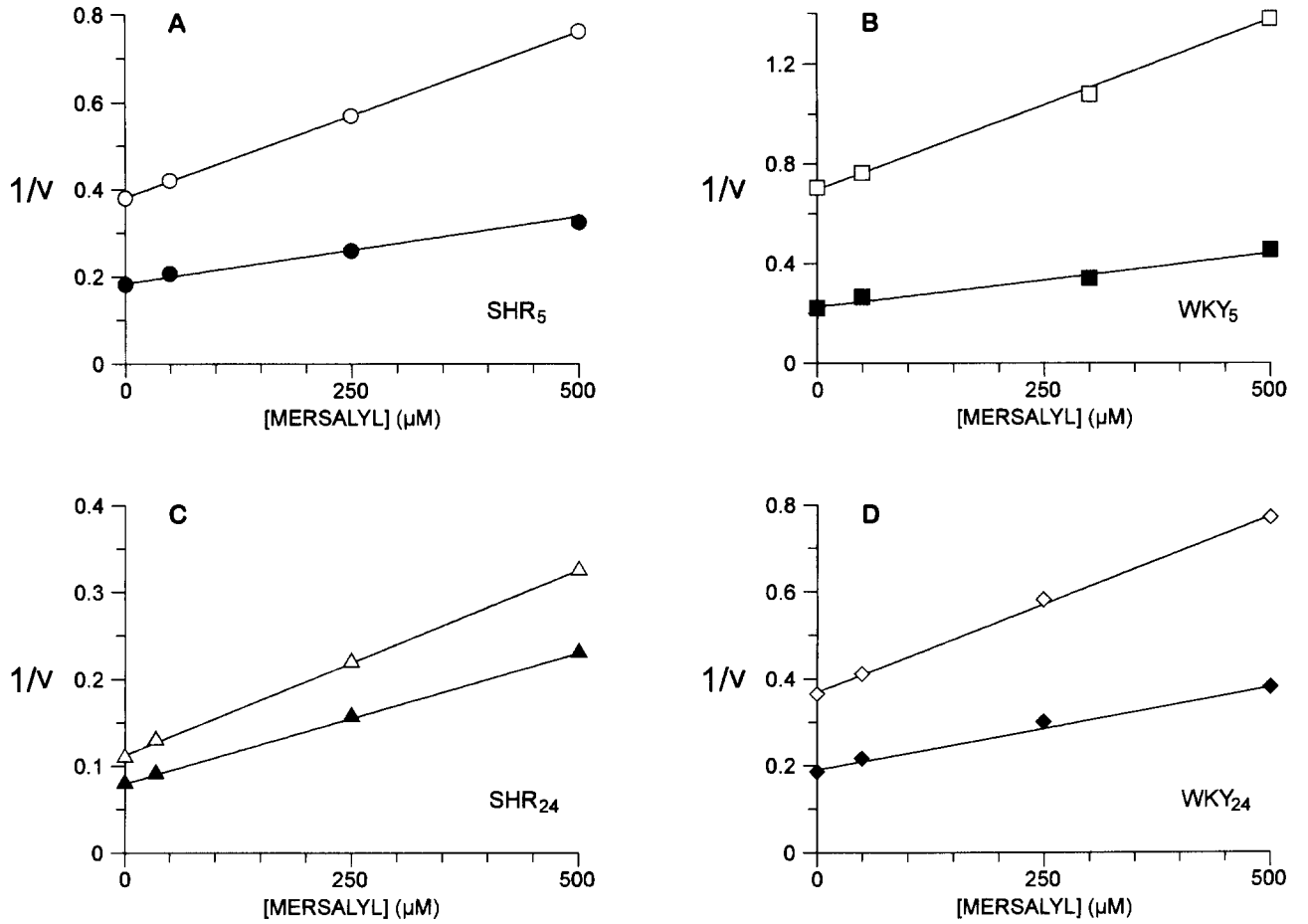

Fig. 4. Dixon plot of the inhibition by mersalyl of the rate of fluorescence increase due to externally added HYP to either 5- and 24-week-old spontaneously hypertensive or Wistar-Kyoto rat heart left ventricle mitochondria. Rat heart left ventricle mitochondria (0.6 mg protein) from either 5- and 24-week-old spontaneously hypertensive (A,C) or age-matched Wistar-Kyoto rats (B,D) were suspended at 20°C for 1 min in 2 ml of incubation medium in the presence of 1.25 μM FCCP and 2 μg ROT (experimental conditions as in Fig. 2). The rate of reduction of intramitochondrial $NAD(P)^+$ was measured by using 1 mM (open symbols) and 10 mM (closed symbols) HYP in the absence or presence of mersalyl at the indicated concentration. The rate is expressed as nmol $NAD(P)^+$ reduced/min per mg mitochondrial protein.

HYP transport rate may be assumed to be an adaptative response of the heart to an imposed work overload [29]. On the other hand, given that the sustained pressure load in spontaneously hypertensive rats is responsible for changes in cardiac function and energy metabolism and, ultimately, in the development of cardiac hypertrophy, adaptation of the heart to a high cardiac work load could lead both to changes in the activity of enzyme systems of myocardial energy production and to new synthesis of structural proteins [30–34], including the mitochondrial carriers. Thus, a change in V_{max} could be due to an increase in the number of carrier proteins (due to genetic alterations), however, a change in the membrane carrier environment, which might increase the specific carrier activity cannot be ruled out. On the other hand, a change in K_m suggests a modification in the substrate binding site, assumed to be consistent with a change in the carrier molecule structure (see mersalyl experiment). Nevertheless, given that non-hemodynamic factors, such as circulating hormones, also represent growth stimuli to cardiac fibroblasts and contribute to structural remodeling of the cardiac interstitium during hypertension, it may be reasonable to suggest a role for these factors in the catabolic pathway of collagen [35]; indeed, collagenase activity was found to be affected by angiotensin II [36,37].

A better understanding of the pathophysiological basis for fibrosis and the mechanisms responsible for fibroblast collagen turnover is of particular importance in hypertension because of the varied alterations in cardiac performance at different stages [38,39], and the possibility of reversing abnormal accumulation of fibrillar collagen with antihypertensive therapy [40–43].

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