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Monoamine Oxidase and Semicarbazide-Sensitive Amine Oxidase Activities in Isolated Cardiomyocytes of Spontaneously Hypertensive Rats

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In the isolated cardiomyocytes of spontaneously hypertensive rats (SHR, 3 months old) MAO A and B activities were significantly increased compared to the myocytes in the hearts of age-matched Wistar–Kyoto rats. This increase was not associated with cardiac hypertrophy in these young animals, but might represent an early event in the development of hypertrophy. A semicarbazide-sensitive amine oxidase (SSAO) activity was found in cardiomyocytes. This activity showed a high affinity for benzylamine (K_m 5–6 μM) and was not inhibited by 10^{-4} M pargyline and 10^{-5} M deprenyl, but was largely inhibited by 10^{-4} M B_{24} (3,5-diethoxy-4-aminomethylpyridine), a specific inhibitor of semicarbazide-sensitive amine oxidase with high affinity for benzylamine. The SSAO enzyme of rat cardiomyocytes is a copper-amine oxidase and has a cross-reactivity with the antibodies raised against pure pig plasma benzylamine oxidase. In the cardiomyocytes of 3-month-old SHR rats the level of this enzymic activity is not significantly increased. © 1997 Academic Press

Key Words: **semicarbazide-sensitive amine oxidase; cardiomyocytes; monoamine oxidase; hypertension.**

Hypertensive heart disease can be defined as the response of the heart to the afterload imposed on the left ventricle by the progressively increasing arterial pressure and total peripheral resistance produced by hypertensive vascular disease.

Hypertension can cause or is related to various cardiac manifestations, among them left ventricular hypertrophy, congestive heart failure, cardiac dysrhythmias, and ischemic heart disease. The potential signals that may induce the development of cardiac hypertrophy have been grouped as growth-promoting hormonal, hemodynamic, vasoconstriction-promoting hormonal, and genetic factors (1).

Spontaneously hypertensive rats (SHR) are a good model for studying hypertensive heart disease. The development of hypertension with cardiac hypertrophy is similar in both humans and SHR (2). This model has served as a means to examine the interrelationship of catecholamines, elevated blood pressure, and cardiac hypertrophy. Changes in the hemodynamic load on the heart are often manifested by the vasoconstrictive action of circulating vasoconstrictors such as angiotensin II and catecholamines. The amine oxidase enzymes such as monoamine oxidase (MAO; monoamine O_2 oxidoreductase, EC 1.4.3.4) and semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6) have an important role in the metabolism of vasoactive substances such as catecholamines, 5-hydroxytryptamine, and histamine (3,4). MAO is an enzyme found predominantly on the outer membrane of the mitochondrion. Two forms of the enzyme, called MAO-A and MAO-B, can be distinguished on the basis of their different relative sensitivities toward inhibition by the acetylenic drugs clorgyline and deprenyl (selegiline). SSAO are enzymes which are resistant to inhibition by millimolar concentrations of acetylenic MAO inhibitors, but are completely inhibited by a 1 mM concentration of semicarbazide, which has little or no activity against MAO (1,5).

Among SSAO enzymes, two might be relevant in

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the development of cardiac hypertrophy: diamine oxidase (DAO) and benzylamine oxidase (Bz.SSAO). This last enzyme is widely distributed in animal tissues and it is predominantly localized in the plasma membrane of the cells (3). DAO has a role in the regulation of the putrescine level in cells, which is a precursor of polyamines that increases in the early stages leading to cardiac hypertrophy (5). Although the physiological role of Bz.SSAO is still unknown, this enzyme has a role in the catabolism of histamine in the rat (6).

Previous studies have reported that MAO activity is high in the heart of SHR compared with normotensive Wistar–Kyoto rats (WKY) (7). This observation was obtained using crude homogenates of the whole heart. Moreover, as far as we know, no data exist concerning MAO and SSAO activity in isolated cardiomyocytes from SHR and WKY rats.

The aim of this current work was to compare MAO and SSAO activity in the isolated cardiomyocytes and verify whether the levels of these enzymic activities are altered in hypertension. The results obtained from this work will provide a base for further studies in the attempt to understand if the variations in the amine oxidizing enzymes might be confirmed in humans and might be considered primary events in the development of hypertrophy.

MATERIALS

Male 3-month-old WKY and SHR were obtained from Charles River (Calco, Lecco, Italy). 7-[^{14}C]-Benzylamine hydrochloride (54 mCi/mmol), 5-hydroxy[*side chain*-2-^{14}C]tryptamine creatine sulfate (55 mCi/mmol), 2-phenyl[1-^{14}C]ethylamine hydrochloride (56 mCi/mmol) and [^{14}C]putrescine dihydrochloride (114 mCi/mmol) were obtained from Amersham (Buckinghamshire, England). Selegiline (Deprenyl) was a gift of Chiesi Farmaceutici Spa (Parma, Italy); pargyline hydrochloride, bovine serum albumin (fraction V) and essentially fatty acid-free bovine serum albumin, collagenase type I, 2,6-dichlorophenolindophenol, and rabbit antiactin-specific antibodies were obtained from Sigma (St. Louis, MO). Anti-rabbit IgG-fluorescein fragment, anti-smooth muscle rat monoclonal antibody, rabbit anti-rat immunoglobulin fluorescein conjugate, NADH, cytochrome c, collagenase, catalase, peroxidase, and dispase were purchased from Boehringer Mannheim Spa (Milan, Italy). The heparin solution was the drug Liquemin Roche. B_{24}(3,5-diethoxy-4-aminomethylpyridine dihydrochloride) was a gift of Professor V. Bertini of Genoa University (Italy). Other reagents were of analytical grade.

METHODS

Preparation of Ventricular Cardiomyocytes

The investigation conforms to the (European Community) rules for the care and use of laboratory animals (86/609/CEE). Animals were kept in our animal facility until they were killed. The weights of the two groups of animals that were used did not significantly differ ($P > 0.05$, Student t test): 237.2 ± 11.8 g (means ± SE of 15 WKY), 247.2 ± 8.0 g (means ± SE of 19 SHR).

Single ventricular myocytes were isolated from 3-month-old SHR and WKY rats, using a protocol based on previously described procedures (8,9). Animals were injected with 500 iu heparin ip and anaesthetized with ether, after which they were killed and the hearts rapidly excised and rinsed in cool low-calcium solution (LCS) containing (mM): NaCl, 120; KCl, 10; KH_2PO_4, 1.2; $MgCl_2$, 1.2; glucose, 10; taurine, 20; and pyruvate, 5. The pH was adjusted to 7.2 with Hepes/NaOH. The heart was mounted in a Langerdoff apparatus and perfused retrogradely with LCS maintained at 37°C and equilibrated with 100% O_2. After 15 min, the solution was quickly changed to LCS plus 1 g L^{-1} collagenase type I, 0.03 g L^{-1} dispase, and 1 g L^{-1} bovine serum albumin (BSA). The time of perfusion with the enzymic solution ranged from 10 to 15 min. In fact, the perfusion was terminated when the heart became soft, and the left and right ventricle including septum were then cut off, chopped into small pieces, and gently stirred in LCS plus 100 μM $CaCl_2$. Dissociated cells were harvested at approximatively 20-min intervals from the tissue by filtration through nylon mesh (200-μm pore size). Those filtrates (generally the second, third, and fourth collected) which contained a high proportion of intact myocytes among the cells were pooled for cell purification. Since the myocytes were larger than nonmyocyte constituents of the heart, the former were isolated and collected by centrifugation (10g for 10 min). An inverted phase-contrast microscope was used to confirm that the cell fraction was enriched in myocytes. The yield of rod-shaped cells ranged from 75 to 85%.

Before performing an enzyme assay on the purified heart cells, it was necessary to wash the cell pellets so as to remove contaminating BSA, which would interfere with protein estimation of cell ho-

mogenates. Consequently, cell pellets were resuspended in 10 mM sodium–potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose (the eventual homogenization medium) and centrifuged at 100*g* for 10 min, and then the same washing and centrifugation steps were repeated. Finally the washed cell pellets were resuspended in a small volume (usually 5 ml) of homogenization buffer.

The homogenate was centrifuged at 600*g* to remove any larger undisrupted particulate matter remaining in the homogenate after cell disruption. The supernatant was centrifuged at 12,000*g* for 20 min to sediment mitochondria. The supernatant was saved and the pellet was washed twice with 10 ml of the same buffer and finally resuspended and kept frozen until use.

SSAO Activity

Benzylamine Oxidase

The Bz.SSAO activity was assayed at 37°C in the 12,000*g* supernatant in the presence of 10^{-4} M pargyline or 10^{-5} M deprenyl, which were incubated for 30 min before the addition of [^{14}C]benzylamine ([^{14}C]Bz), as already described (10,11). The 10^{-4} M B_{24} was used in every sample to demonstrate that the measured activity was an SSAO activity with high affinity for benzylamine. This inhibitor was also preincubated for 30 min.

The reaction mixture contained either 0.1 ml of 0.067 M sodium–phosphate buffer, pH 7.4, or 0.1 ml of 12,000*g* supernatant, 0.05 ml of catalase (43 U/ml), 0.05 ml of the same buffer, or 0.05 ml of the same buffer containing the inhibitors. After 30 min of preincubation, [^{14}C]benzylamine was added and the reaction was carried out for 60 min. The reaction was then stopped with 0.1 ml of 3 *N* hydrochloric acid and the aldehyde formed was extracted with 1 ml of ethylacetate. Then 0.5 ml of ethylacetate in 10 ml of Instagel was counted in a Packard liquid scintillation counter. Each measurement was carried out in duplicate. It was previously shown that the reaction was linear for 90 min; therefore a time of 60 min was used to better estimate the enzymatic activity. Different [^{14}C]Bz concentrations (0.41–0.83–1.66–3.33–5.00–8.33 μM) in the presence of 10^{-4} M pargyline were used in order to determine the kinetic constants. Michaelis constants were obtained according to Wilkinson (12). All samples were also tested with a saturating concentration of [^{14}C]Bz (83 μM) in the absence and in the presence of 10^{-4} M pargyline, 10^{-5} M deprenyl, or 10^{-4} M B_{24}. The sum of the activity obtained in the presence of pargyline or deprenyl and those obtained in the presence of B_{24} was always checked; it gave the total activity obtained in the absence of inhibitors within the limits of experimental errors. When histamine was used as competitive inhibitor of [^{14}C]Bz oxidation it was added to the incubation mixture with the substrate.

Diamine Oxidase

DAO activity was assayed at 37°C in the 12,000*g* supernatant using 3×10^{-4} M [^{14}C]putrescine as substrate. The mixture contained 0.1 ml of supernatant and putrescine in a total volume of 0.4 ml of 0.067 M sodium–phosphate buffer, pH 7.4. The reaction was blocked and radioactivity measured as described (13).

Monoamine Oxidase Activity

MAO A. This activity was assayed in the mitochondrial pellet at 37°C using 0.16 mM [^{14}C]5-hydroxytryptamine (5-HT) by following the reaction for 15 min. Better experimental conditions were preliminarily established with the mitochondria of WKY hearts: high concentrations of 5-HT did not inhibit the enzyme; therefore, the final concentration of 166 μM was selected, the activity was linearly related to the enzyme concentration, and the reaction was linear for 25 min of incubation time.

The mixture contained 0.05 ml of mitochondrial suspension, 0.05 ml of 1 mM [^{14}C]5-HT in a total volume of 0.3 ml obtained with 0.067 M sodium–potassium phosphate buffer, pH 7.4. After 5 min of preincubation the reaction was followed for 15 min and stopped with 0.1 ml of 3 *N* hydrochloric acid, and the aldehyde was extracted with 1 ml of ethylacetate. Then 0.5 ml of ethylacetate were counted as described in the SSAO assay. The blank was obtained by adding 0.1 ml of 3 *N* hydrochloric acid before the addition of the substrate.

MAO B. The activity was assayed at 37°C in the mitochondrial pellets. Preliminary experiments with WKY heart mitochondria have shown that high concentrations of β-phenylethylamine (β-PEA) inhibit the activity. Therefore, a concentration of 33 μM was selected. It was also shown that the reaction was linear for only 15 min, so a 5-min incubation time was selected. The reaction mixture was identi-

cal to those of MAO A in which β-PEA instead of 5-HT was used.

Protein Determination

Proteins were evaluated using the method of Lowry *et al.* (14) with bovine serum albumin as the standard.

NADH-Cytochrome c Reductase

This activity was assayed according to King (15) at 37°C. All assays were performed in triplicate.

Succinate-Cytochrome c Reductase

This activity was assayed according to Phillips and Langdon (16) at 37°C and all assays were performed in triplicate.

Immunofluorescent Histochemistry

The immunofluorescence histochemistry of isolated cardiomyocytes was carried out according to Buffoni *et al.* (17). SSAO was stained using the purified polyclonal antibodies obtained in the rabbit against the pig plasma benzylamine oxidase as the first antibody. The fluorescent second antibody was fluorescent antirabbit-globulin. The presence of SSAO was studied in parallel with the presence of α-actin and smooth muscle proteins.

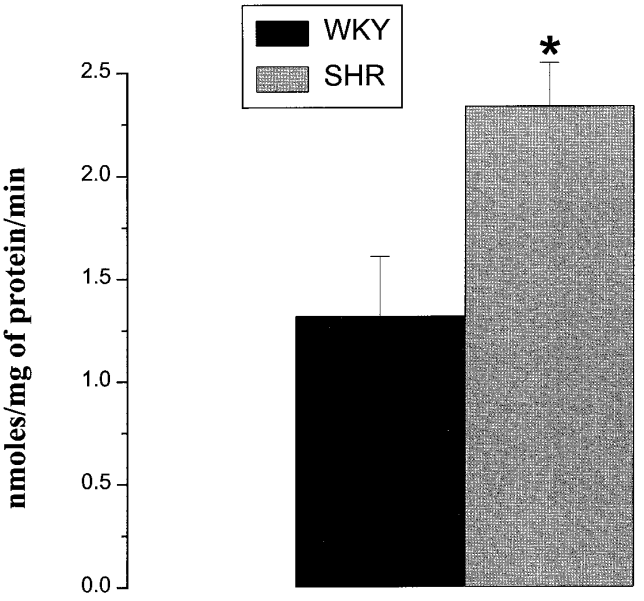


FIG. 1. Mitochondrial monoamine oxidase MAO A activity of isolated myocytes of SHR and WKY hearts; rate of deamination of [^{14}C]5-hydroxytryptamine. Values are the mean ± SE of isolated myocytes of 12 WKY and 15 SHR hearts. *The difference between this value and the value obtained in the WKY group is statistically significant ($P < 0.05$) (Student t test).

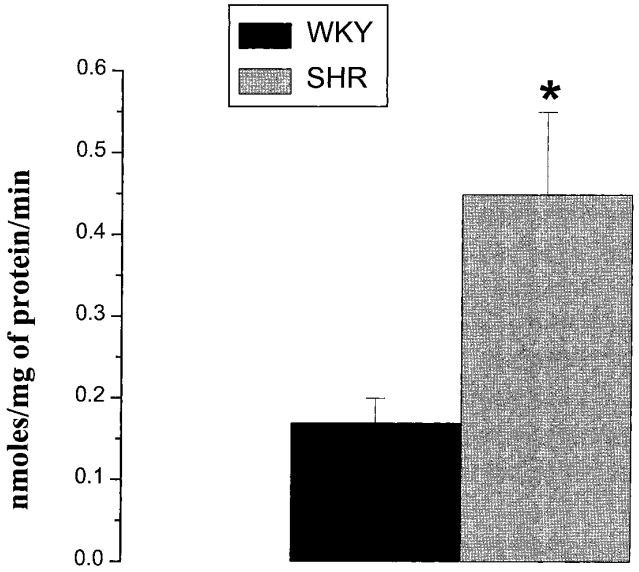


FIG. 2. Mitochondrial monoamine oxidase MAO B activity of isolated myocytes of SHR and WKY hearts; rate of deamination of [^{14}C]phenylethylamine. Values are the mean ± SE of isolated myocytes of 12 WKY and 15 SHR hearts. *The difference between this value and the value obtained in the WKY group is statistically significant ($P < 0.05$) (Student t test).

RESULTS

Heart Weight

The weights of the hearts of the two groups of animals did not significantly differ ($P > 0.05$; Student t test): 1.09 ± 0.06 g (means ± SE of 15 WKY), 1.19 ± 0.07 g (means ± SE of 19 SHR). The ratio (weight of the heart/weight of the animal) of the two groups did not significantly differ ($P > 0.05$; Student t test): 0.0045 ± 0.0002 (means ± SE of 15 WKY), 0.0048 ± 0.0002 (means ± SE of 19 SHR). Therefore, these young rats had not yet developed an evident cardiac hypertrophy.

MAO Activity

A statistically significant increase in the activity of both MAO A and B was found in SHR cardiomyocytes compared to the WKY cardiomyocytes (Figs. 1 and 2), whereas no significant differences were found

TABLE 1

NADH-Cytochrome c Reductase (NADH cyt-r) and Succinate-Cytochrome c Reductase (Succ-cyt-r) in the Mitochondria of Cardiomycytes of SHR and WKY Rats

	NADH cyt-r (nmol/mg/min)	Succ-cyt-r (nmol/mg/min)
WKY	116.0 ± 25.5	72.3 ± 18.6
SHR	126.7 ± 31.0	43.8 ± 5.2

Note. Values are the mean ± SE of isolated myocytes of 10 WKY and 10 SHR hearts. The differences between the values are not statistically significant ($P > 0.05$) (Student t test).

in the level of activity of NADH-cytochrome c reductase and succinate-cytochrome c reductase (Table 1).

SSAO Activity

The 12,000*g* cardiomyocytes supernatant had low SSAO activity toward Bz. The percentage of the total activity in the presence of 10^{-4} M pargyline was 53.2 ± 3.82 (means ± SE of 20 determinations in duplicate); in the presence of 10^{-5} M deprenyl, 53.0 ± 3.31 (means ± SE of 22 determinations); in the presence of 10^{-4} M B_{24}, 60 ± 2.47 (means ± SE of 20 determinations). These results clearly indicated that the Bz.SSAO activity of cardiomyocytes represented approximatively 50% of the total activity toward Bz. In fact, in the rat heart Bz can be deaminated by both forms of MAO (A and B) and by Bz.SSAO (12,23,24). The Bz.SSAO activity was always assayed in the presence of 10^{-4} M pargyline, a concentration able to inhibit both forms of MAO.

Figure 3 shows that the level of this activity in the SHR heart myocytes is not significantly different from the level found in the myocytes of matched WKY. This enzymic activity shows a very high affinity for Bz in both groups (Fig. 4). No significant dif-

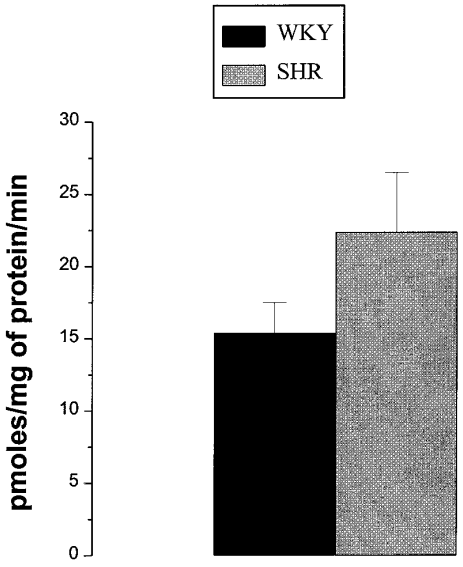


FIG. 3. Semicarbazide-sensitive amine oxidase in isolated myocytes of SHR and WKY hearts; rate of [^{14}C]benzylamine deamination. The activity was assayed after 30 min of incubation with 10^{-4} M pargyline. Values are means ± SE of isolated myocytes of 15 WKY and 19 SHR hearts. The difference between the values is not statistically significant ($P > 0.05$) (Student t test).

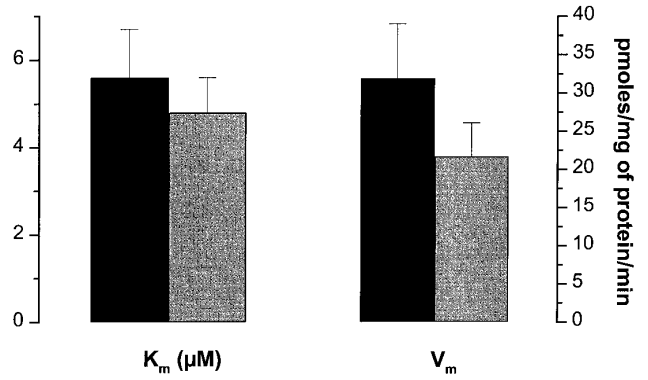


FIG. 4. Semicarbazide-sensitive amine oxidase in isolated myocytes of SHR and WKY hearts; kinetic constants. The activity was assayed after 30 min of incubation with 10^{-4} M pargyline. Values are means ± SE of isolated myocytes of 9 WKY and 11 SHR hearts. The difference between the values is not statistically significant ($P > 0.05$) (Student t test).

TABLE 2

Inhibition of the Oxidation of Two Concentrations of [^{14}C]benzylamine by 1 mM Histamine in the Presence of 10^{-4} M Pargyline by the 12,000*g* Supernatant of Cardiomyocytes of SHR and WKY Rats

	% Inhibition [^{14}C]benzylamine	
	8.3 μM	16.6 μM
WKY	46.2 ± 3.2	35.8 ± 5.2
SHR	44.6 ± 4.8	33.0 ± 4.5

Note. Values are the mean ± SE of isolated myocytes of four WKY and four SHR hearts analyzed in duplicate. The differences between the values are not statistically significant ($P > 0.05$) (Student t test). The myocytes of eight SHR hearts and seven WKY hearts were assayed for the DAO activity (see Methods): no DAO activity was observed.

ferences were observed between the K_m and V_m of the two groups.

No DAO activity was detected in the SHR and WKY cardiomyocytes, but histamine was a good competitive inhibitor of [^{14}C]Bz ozidation (Table 2), indicating that histamine is a substrate of this enzyme as described for other rat tissues (1).

Immunofluorescence Histochemistry

The treatment of myocytes with the antibodies raised against pure pig plasma benzylamine oxidase clearly showed the presence of the SSAO, which cross-reacted with this antibody and appeared mainly localized on the surface of the cells. The antibody against α-actin indicated the presence of this substance inside the cells. The myocytes gave a good fluorescence also with the monoclonal antibody (IgM class) anti-smooth muscle cells (Fig. 5). The shape of the cells in the figure was altered by the fact that the cells were frozen and then striped on the glass slide.

DISCUSSION

Previous studies have reported that MAO activity is high in the hearts of SHR compared with those of normotensive WKY using kynuramine (18) and 5-HT, tyramine, and β-PEA as substrates (7), whereas no change in Bz oxidation was observed. These observations were obtained using crude homogenates of the whole heart. Moreover, as far as we know, no data exist concerning MAO and SSAO activity in isolated cardiomyocytes from SHR and WKY rats.

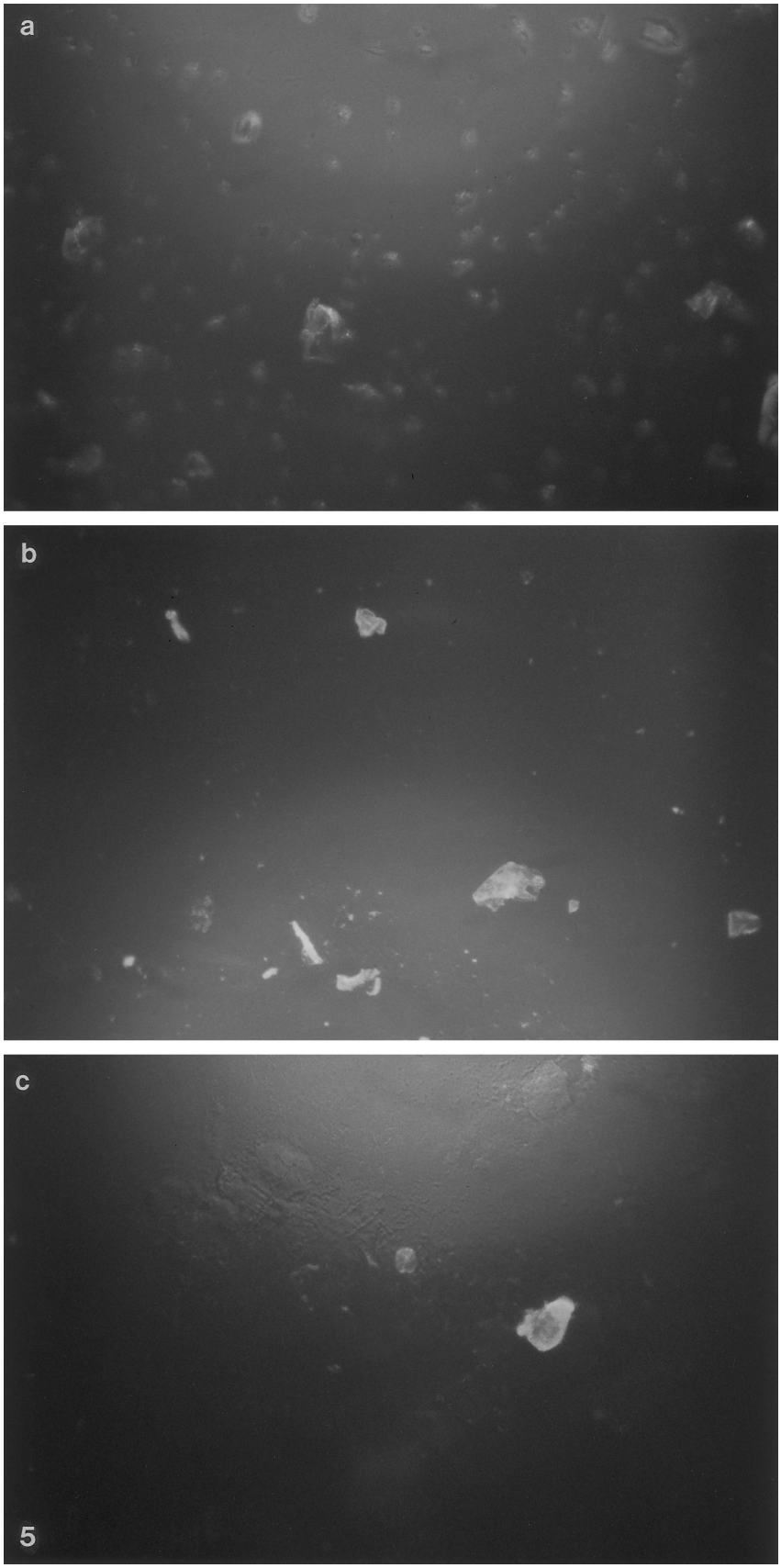
The specific activity of both MAO A and B is significantly higher in the cardiomyocytes obtained from SHR compared to WKY rats. Different factors could be responsible, such as an increase in the amount of enzyme protein or changes in the enzyme properties, rate of enzyme degradation, or the mitochondrial membrane. No statistically significant differences were observed in the specific activity of NADH and succinate cytochrome c reductases. It is therefore unlikely that a gross change in the number of mitochondria and in the structure of the mitochondrial membrane is involved since succinate cytochrome c reductase on the inner membrane and NADH cytochrome c reductase on the inner and outer mitochondrial membranes are both unaffected in these 3-month-old SHR.

It is known that MAO of the heart exhibits increasing activity with increasing age and weight of the heart (19), with adrenalectomy (20) and following treatment with thyroid hormone (21,22). The study of MAO activity of the heart of several species of animals (mouse, guinea pig, rabbit, and cat) has shown that the rat is the only species possessing the peculiar quality of increasing activity with age and weight (19). It is not yet clear if the observed variations are due to an increase in enzyme synthesis or to a decrease in enzyme degradation. On the other hand, all these studies have been carried out on whole heart homogenates. In this work we have shown that the increase in MAO activity is present in cardiomyocytes of SHR hearts and this increase is not due to an increase in the heart weight.

The MAO B activity is much lower than the MAO A activity, but in SHR the increase in MAO B activity is much higher than that of MAO A. Cardiomyocytes also express an SSAO activity with a high affinity for Bz, the level of which is nearly 100 times lower than the level of MAO A activity and 10 times lower than that of MAO B activity. Both level and kinetic properties of this enzymatic activity are not changed in SHR myocytes. No age-related change in this activity has been seen in the rat heart (23), whereas a decrease in this enzymatic activity has been described in the atria of 5-month-old SHR (24). In 7-month-old SHR hearts no variation in the specific activity of SSAO were observed (25), whereas this enzymatic activity was increased in blood vessels (25). The physiological role of this enzyme is still under investigation (3,4). In some species histamine may be a physiological substrate. We have shown that in the mesenteric arterial bed of the rat, SSAO with a high affinity for Bz is the only enzyme present which is able to deaminate histamine (6). Reduced vascular histamine content and a increased rate of histamine deamination have been described in SHR (26).

SHR are considered a suitable laboratory counter-

FIG. 5. Immunofluorescence histochemistry of cardiomyocytes using antibodies to pig plasma amine oxidase, to actin, and to smooth muscle proteins. As described under Methods, the second antibody was always a fluorescein-labeled antibody, diluted 1:10. (a) Fluorescence obtained using purified rabbit polyclonal antibodies against the pure pig plasma benzylamine oxidase (7 mg/ml) as first antibody; (b) fluorescence obtained using rabbit anti-actin antibody as first antibody (1:10); (c) fluorescence obtained using rat anti-smooth muscle monoclonal antibody (1:10). Original amplification, $16 \times 1.25 \times 8 \times 4.16$.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part to human essential hypertension. SHR develop left ventricular hypertrophy in response to slow progressive elevation in arterial pressure and total peripheral resistance (27).

Hypertensive rats of 3 months of age are considered young. Since hypertrophy of the mammalian heart is a time-dependent process which evolves continuously during the life span of the animal, these animals are probably in an early phase of the development of cardiac hypertrophy. Because of this, we were not able to reveal any difference in the heart weight between WKY and SHR hearts at this age, whereas the difference is statistically significant between 18-month-old WKY and SHR (28).

Because no DAO activity was found in myocytes, MAO B has a role in the degradation of tele-methylhistamine (29), the other route of histamine degradation. The observed increase in MAO B activity may increase the rate of histamine degradation which has been described in SHR rats (26).

An early step in the development of cardiac hypertrophy is an increase in ornithine-decarboxylase activity with a consequent increase in polyamines and putrescine (5). Diamine oxidase as well as MAO and SSAO are involved in the control of the putrescine level. In fact, MAO and SSAO are able to oxidize acetylputrescine and the acetylation followed by oxidative deamination is a route of putrescine catabolism. The observed increase in MAO activity in SHR myocytes might be an early expression of an increase in putrescine and the determination of the amine oxidase activities could be an early marker for the development of cardiac hypertrophy.

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