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SUBCELLULAR EVALUATION OF SUBCHRONIC TOXICITY OF SALICYLHYDROXAMIC ACID (SHAM) IN RATS: EX-VIVO CHANGES IN RESPIRATORY RATE OF LIVER MITOCHONDRIA AND IN HEPATO RENAL LYSOSOMAL ENZYMES

BY

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ABSTRACT

SHAM was given orally daily for 5 days per week along three months in dose levels 200 and 500 mg/kg to adult male rats. Subsequently, the required procedures were taken for obtaining the purified mitochondrial fraction, on which respiratory rates of O_2-uptake were estimated by the warburg technique.

In addition, the total activity contents of four selected lysosomal hydrolytic marker enzymes namely : acid phosphatase "ACP", N-acetyl-B-glucosaminidase "B-NAG", B-galactocidase "B-GAL" and α-glactosidase " α-GAL" as well as the respective rates of their extralysosomal release were evaluated in the properly obtained hepatic and renal fractions.

The rates of aerobic mitochondrial respiration revealed a highly significant activation in O2-consumption under low dose of SHAM, while the high dose exerted a marked reduction in O_2-uptake.

Subchronic exposure to SHAM resulted in a dose-dependent enhancement in hepatic lysosomal enzymatic activity of the investigated four marker enzymes. The renal lysosomal contents of these enzymes were markedly reduced. The rates of hepatic extralysosomal enzymatic release were reduced indicating a stabilizing effect on the lysosomal membrane under the used doses, while in case of renal lysosomal membranes, the effect of SHAM varied greatly with variation of the dose level.

The obtained results will be discused in view of their relevent therapeutic and toxicological repercussions.

INTRODUCTION

It is known since more than two decades that studies on lysosomes may provide general clues to the mechanisms of drug action. The functions of lysosomes are varried, and therefore a given pharmacologic agent can affect the lysosomal system in many ways (De Duve 1968, Weisman et al., 1969).

On the other hand, mitochondria are the key sites of the enzymes of the respiratory chain. The study of drug effects on mitochondria can contribute to understanding of the possible drug side effects.

Thus, there was a need for the investigational drug (SHAM) to be evaluated for its possible effects on the oxygen consumption of rat liver mitochondria and on hepato-renal lysosomal enzymes.

MATERIALS AND METHODS

MATERIALS:

Test drugs: SHAM is salicylhydroxamic acid, it contains not less than 99% and not more than 100.5% $C_7H_7NO_3$. It was supplied by El-Nasr Co, Cairo Egypt. The substrates and other chemicals were purchased from Sigma chemical Co.

METHODS:

Animal Husbandary and Drug Administration:

Male albino rats, weighing 140-160 gm were used. They were fed on a standard laboratory diet and watered ad libitum. SHAM was given orally daily for 5 days per week along three months in dose levels 200 and 500 mg/kg.

Isolation of Rat Liver Mitochondria:

Livers of adult male albino rats were used for isolation of pure mitochondrial suspensions by differential centrifugation as stated by Campello et al., (1970) and De Robertis et al., (1962).

Oxygen Uptake Measurements:

The system of oxygen uptake of rat liver mitochondria was that of Warburg techniques described by Thimnappayya et al., (1970) and Barcina et al., (1986).

Isolation of Hepatic Lysosomal Fractions:

After animal decapitation, liver was quickly removed and homogenized in cold 0.25 M sucrose in volumes of 17% (w/v) homogenate. Medium-homogenization was carried out by teflone pestle. After filtration through three sheets of gauze, the homogenate was centrifuged at 27000 r.p.m. for 15 min. by a subsequent differential centrifugation, the lysososomal fraction was obtained as

stated by Tanaka and Iisuka (1968).

Preparation of Renal Lysosomal Enzymes:

After animal decapitation, kidneys were removed quickly, the renal cortical tissue was quickly separated weighed and minced. The subsequent entire homogenization and isolation procedure was carried out at 0-5C unless otherwise stated according to the method of Powell and Reidenberg (1982).

Methods of Liver Lysosomal Enzymatic Activity Assays:

The activity of the four marker acid hydrolases has been measured according to the methods described by Van Hoof and Hers (1968) by using the following substrates.

Orthophosphoric Monoester Phosphohydrolase (EC. 3.I.3.2.):

"ACP" p-nitrophenyl phosphate sodium salt pure (Koch-light)

N-Acetyl-B-D-glucosaminidase (EC.3.2.I.30):

"B-NAG" p-nitrophenyl 2-acetamido-2-deoxy-B-D-glucopyranoside (Koch-light).

B-galactosidase (EC.3.2.I.23):

"B-GAL" p-nitrophenyl-B-D-galactopyranoside (Koch-light)

α-Galactosidase (EC. 3.2.I.22):

"α-GAL" p-nitrophenyl α-D-galactopyranoside (Koch-light).

Statistical Methods:

Levels of significance of differences between means of medicated and control batches were statistically evaluated by use of the non-paired student's "t" test (Goldstein, 1969).

RESULTS

As evident from comparative appraisal of the total O2-consumption values for control non-medicated samples, the first hour O2-uptake was lower than second hour estimate. Exposure to high dose of SHAM resulted in marked reduction by 15% at 30 min and to 22% over 2 hours incubation in O2-consumption. Low dose of SHAM exerted well pronounced magnitude of very highly significant activation of total O2-consumption by an initial values amounting to 175% over 30 min declining down to a substantially low intensity of increase (56%) over 120 min incubation (Table 1).

The results in table (2) demonstrated markedly enhanced liver lysosomal enzymatic activity of the four marker enzymes isolated from adult normal male albino rats under the influence of applied two dose levels of SHAM. The effect was dose-dependent in case of all studied enzymes.

The data in table (3) indicated that the rates of extralysosomal release were markedly reduced in the case of B-NAG, B-GAL and α-GAL under influence of the low dose level. The high dose level of SHAM exhibited appreciable reductions in the case of ACP, B-NAG and α-GAL.

Measurement of the total activities of four renal lysosomal enzymes isolated from adult control male albino rats after treatment by the high dose of SHAM revealed slight reductions in the case of ACP and α-GAL while total B-NAG and B-GAL enzymatic assay values were not altered. In the meantime, high dose of SHAM induced a significant decrease in the total enzymatic activity of B-NAG and ACP (Table 4).

Table (5) reveals that the extralysosomal release rates of

Table (1) : EFFECT OF SHAM ON THE OXYGEN CONSUMPTION OF ISOLATED RAT LIVER MITOCHONDRIAL SUSPENSIONS over TWO HOURS INCUBATION PERIODS. (n = 6).

Items	O_2-consumption as recorded over specified incubation periods (u Mol. O_2/mg protein) ($\bar{X} \pm$ S.E.M.).			
	30 min.	60 min.	90 min.	120 min.
Control	0.072 ± 0.004	0.159 ± 0.001	0.231 ± 0.0002	0.286 ± 0.0005
Low dose 0.2 gm/kg/day.	0.198 ± 0.0001 ↑ + 175%***	0.304 ± 0.0003 ↑ + 90 %***	0.401 ± 0.0007 ↑ + 74 %***	0.447 ± 0.0007 ↑ + 56 %***
High dose 0.5 gm/kg/day	0.061 ± 0.0003 ↓ - 15 %**	0.134 ± 0.0003 ↓ - 16 %**	0.169 ± 0.001 ↓ - 27 %***	0.224 ± 0.0003 ↓ - 22 %***

Statistical significance: ** $P < 0.01$ $P < 0.001$

Table [2] : EFFECT OF SHAM ON THE TOTAL ACTIVITIES OF HEPATIC LYSOSOMAL ENZYMES FOLLOWING THREE MONTHS TREATMENT IN ADULT RATS. (n = 6).

Items	Specific total enzymatic activities (n mole/mg protein/hr.) ($\bar{X} \pm$ S.E.M.)			
	ACP	β-NAG	B-GAL	α-GAL
control	1263 ± 41.85	723 ± 13.20	355 ± 8.92	84 ± 2.91
Low dose 0.2 gm/kg/day	1433 ± 9.63 ↑ + 14 %**	797 ± 3.69 ↑ + 10 %***	364 ± 3.69 ↑ + 2.5 % †	103 ± 2.41 ↑ + 23 %***
High dose 0.5 gm/kg/day	3119 ± 45.11 ↑ + 147 %***	1737 ± 2.75 ↑ + 140 %***	688 ± 2.79 ↑ + 94 %***	232 ± 0.32 ↑ + 176 %***

Statistical significance : *** Significant at $P < 0.001$
** Significant at $P < 0.01$
† Insignificant

Table [3] : EFFECT OF SHAM ON THE EXTRALYSOSOMAL RELEASE OF HEPATIC LYSOSOMAL ENZYMES FOLLOWING THREE MONTHS TREATMENT IN ADULT MALE RATS (n=6).

Items	Specific enzymatic release activities (n mole/mg protein/hr.) ($\bar{X}$ + S.E.M.)			
	ACP	β-NAG	B- GAL	α-GAL
Control	693 ± 14.429	321 ± 2.523	121 ± 2.904	32 ± 1.261
Low dose 0.2 gm/kg/day.	686 ± 6.583 ↓ - 1 % †	263 ± 1.722 ↓ - 18 %***	83 ± 1.265 ↓ 31 %***	20 ± 0.836 ↓ - 38 %***
High dose 0.5 gm/kg/day.	571 ± 13.374 ↓ - 18 %***	297 ± 10.791 ↓ - 8 %*	134 ± 5.514 ↑ + 10 %*	20 ± 0.730 ↓ - 38 %***

Statistical significance : *** Significant at $P < 0.001$
** Significant at $P < 0.01$
† Insignificant.

ACP : Acid Phosphatase.
B-Gal : B-Galactosidase.
β-NAG : N-acetyl B-D-glucosaminidase.
α-GAL : α-Galactosidase.

Table [4] : EFFECT OF SHAM ON THE TOTAL ACTIVITIES OF RENAL LYSOSOMAL ENZYMES FOLLOWING THREE MONTHS TREATMENT IN ADULT MALE RATS. (n = 6).

Items	Specific enzymatic total activities (n mole/mg protein/hr.) ($\bar{X} \pm$ S.E.M.)			
	β-NAG	ACP	B-GAL	α-GAL
Control	2183 ± 65.63	1606 ± 9.32	504 ± 11.02	476 ± 5.18
Low dose 0.2 gm/kg/day	2116 ± 45.26 ↓ - 3 % †	1434 ± 11.02 ↓ - 11 %***	505 ± 8.68 ↑ + 0.3 % †	441 ± 4.78 ↓ - 7 %**
High dose 0.5 gm/kg/day	1966 ± 12.42 ↓ - 10 %**	921 ± 13.52 ↓ - 43 %***	503 ± 10.71 ↓ - 0.3 % †	460 ± 3.26 ↓ - 3 % †

Statistical significance : *** Significant at $P < 0.001$
** Significant at $P < 0.01$
† Insignificant.

ACP : Acid Phosphatase.
B-Gal : B-Galactosidase.
β-NAG : N-acetyl B-D-glucosaminidase.
α-GAL : α-Galactosidase.

Table [5] : EFFECT OF SHAM ON THE EXTRALYSOSOMAL RELEASE OF RENAL LYSOSOMAL ENZYMES FOLLOWING THREE MONTHS TREATMENT IN ADULT MALE RATS (n=6)

Items	Specific enzymatic release activities (n mole/mg protein/hr.) (X ± S.E.M.)			
	β-NAG	ACP	B-GAL	α-GAL
Control	869 ± 9.26	721 ± 4.38	259 ± 2.21	124 ± 1.26
Low dose 0.2 gm/kg/day.	1006 ± 3.89 ↑ + 15 %***	865 ± 3.08 ↑ + 20 %***	276 ± 2.12 ↑ + 7 %***	132 ± 1.78 ↑ + 6 %**
High dose 0.5 gm/kg/day.	685 ± 1.46 ↓ - 21%***	590 ±10.85 ↓ - 18 %***	212 ± 1.64 ↓ - 18 %***	92 ± 0.94 ↓ - 26 %***

Statistical significances : *** Significant at $P < 0.001$
** Significant at $P < 0.01$

the four test marker renal enzymes were increased by variable magnitudes under the influence of low dose of SHAM. The peak augmentation in the release rates was recorded by ACP (20%) followed in descending order by B-NAG (15%), B-GAL (7%) and α-GAL (6%).

In contrast, high dose of SHAM elicited a decrease in the extralysosomal release rates of the four test marker renal lysosomal enzymes.

DISCUSSION

The findings of the current study demonstrates that the high dose of SHAM inhibited O2-uptake of rat liver mitochondria, while the low dose accelerated the O2-consumption of the mitochondria (Table I). The acceleration of O2-uptake may be due to an ability of SHAM to increase the synthesis along with concomitant enhancement of the activity of a diversity of cellular enzymes and coenzymes notably so far lactate dehydrogenase "LDH", promoting the entry of the lactate and pyruvate and products of the glycolytic cycle from the extramitochondrial space into the intramitochondrial matrix (Rhodes, 1969). It is also most likely that SHAM likewise increases synthesis of enzymes exerting regulatory control on the specific translocation mechanism and exchange of the adenine nucleotides viz ADP and ATP across the mitochondrial membrane.

The during incubation observed decline in the magnitude of increase in O2-consumption rate of mitochondrial suspension exposed to low dose during incubation may be due to a competitive inhibition of the free influx of ADP into the mitochondrial matrix. Such a competitive inhibition may be attributed to accumulation of excess ATP generated in the course of accelerated oxidative phosphorylation with consequent suppression of the larger portion of enhanced O2-uptake rate achieved by the adenine dinucleotide (Klingenberg and Pfaff (1968). On contrast, the high dose of SHAM exerted highly significant reduction. This finding may indicate an ability of SHAM to undergo metabolic biotransformation in rat liver mitochondria via reduction precluding an appreciable proportion of the available H (proton) from an appreciable proportion of the available H (proton) from participation in the electron transport chain sustaining mitochondrial O2-utilization, the matter which needs further investigations.

The effects of SHAM on respiration may be partly mediated through changes in fatty acid composition, which have an additive effect on protein synthesis and respiratory chain components as described by Vaddanahally et al. (1986) who explained that phospholipids and fatty acids as active components of biological membrane, contribute in a wide variety of biological phenomena such as activation of membrane-bound enzymes (Nemecz, et al., 1981 and 1986) and regulation of transport processes (Solomon et al., 1983 and Yorck et al., 1984).

It can be also suggested that the inhibition of respiration in rat hepatic mitochondria by the high dose of SHAM may be due to the interaction with either the NADH dehydrogenase complex or other NADH-dependent enzymes. Such proposals are based on the finding of Cameron et al. (1986).

The two applied doses of SHAM exerted highly significant increase in total enzymatic activities of the four studied hepato-lysosomal enzymes reflecting enhanced rates of enzymatic synthesis. The extralysosomal release rate of these specified enzymes were in the meantime markedly decreased by values surpassing the corresponding initial basal estimates.

On basis of the striking features that emerged from the present biochemical investigations it may be justifiably that SHAM do not evoke any deleterious side-effects by the two doses of the experiment.

The effects of SHAM on the activity of lysosomal enzymes may be due to the hydroxamic acid binding to the enzyme in the same manner as a typical substrate. This binding site is "close to", but not directly "on to". Thus, the mode of the exerted inhibition on lysosomal enzymes by SHAM may be due to metal chelation, and/or mechanisms involving either H bonding at the reducing substrate binding site or the formation of a chargetransfer complex between hydroxamic acid and an electron-accepting group in the enzyme. Such like proposals are considered feasible, as stated by Rich Peter et al. (1978).

The inhibition of the lysosomal enzymes release through the membrane under the effects of SHAM means a stabilizing effect of the drug on membrane permiability. This effect may be due to the phenolic ring and hydroxyl groups in the chemical structure of SHAM. Similar proposals were suggested by Teleb (1978) for the stabilizing effect of oestrogen which was probably due to the phenolic ring and the hydroxyl groups in their structures.

On the other hand administration of the two dose levels of SHAM exerted a marked decrease in the total enzymatic activity contents for the 4 studied renal lysosomal enzymes. The low dose of the drug revealed a labilizing effect on the renal lysosoamal membrane permiability while the high dose of drug showed a stabilizing effect on the membrane by decreasing the extralysosomal release for the selected marker renal lysosomal enzymes.

These finding indicated that, the selectivity in the occurence of markedly enhanced rates of extralysosomal release of the renal lysosomal enzymes under the effects of low dose of SHAM and the inhibitory effect of the high dose on the lysosomal enzymes of kidney cells could be ascribed partly to the deficiency of the cytoprotective influence of prostaglandine and related prostanoids created by the appraciable shortage in the arachidonic fatty acid (Peskar et al., 1984).

A deleterious effect on the kidney was observed probably due to the accumulation of drug metabolites like salicylic acid and hydroxylamine in the renal tissue (Hamed et al., 1990).

Hydroxylamine is known to increase chloride ion secretion in the dog colon (Rangachari and McWade, 1987), inhibit human platelet aggregation (Iizuka and Kugawa, 1972), stimulate Na, K, ATP-ase activity (Sachs et al.., 1971), and exert an inactivating effect on a number of coenzymes and intermediates of cell metabolism by reacting chemically with carbonyl and ester groups (Budowsky, 1976).

Thus, through the present work highlights on the subcellular effects of SHAM have been elucidated for the first time. The obtained findings point to the need for additional in-vitro studies.

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