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PARADOXICAL EFFECT OF LEUPEPTIN IN VIVO ON CATHEPSIN B ACTIVITY

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Leupeptin is a potent inhibitor of cathepsin B in vitro and is presumed to act in a similar manner in vivo. It is currently being used in several laboratories to examine the role of lysosomal proteinases such as cathepsin B in mouse models of muscular dystrophy. This report clearly demonstrates that leupeptin in adequate concentrations in vivo, is a potent stimulator of cathepsin B activity in striated muscle, heart, liver and kidney of the mouse. This paradoxical effect indicates that care is required in the interpretation of the results of the use of leupeptin as a cathepsin B inhibitor in vivo and that its use as an antiprotease for therapeutic purposes may be limited. Studies on CBZ-Phe-Ala-CHN_2 demonstrated that this agent, when administered in vivo, inhibited Cathepsin B in the tissues assayed.

Our laboratory has been studying the in vivo potential of anti-proteases to inhibit in situ lysosomal proteases which may be involved in a variety of pathological processes caused by discharge of the lysosome or leakage of the lysosomal membrane. We have reported that pepstatin and analogues can gain entrance into a variety of tissues and inhibit tissue cathepsin D for prolonged periods lasting for 6 days (1). Host response, however, to tumor causes an apparent enhancement or induction of cathepsin D enzyme activity which shortens the duration of the effectiveness of the anti-protease to maintain inhibition at high levels.

Our current report concerns the intracellular protease cathepsin B and attempts to define the "kinetics" of inhibition following administration of leupeptin, and CBZ-Phe-Ala-$CH-N_2$ (Z...), known inhibitors of cathepsin B and related enzymes (2, 3). We wish to report that while the Z... compound appears to be a reasonable inhibitor of cathepsin B in vivo, leupeptin paradoxically stimulated cathepsin B activity, particularly in the heart and striated muscle, although the activities in liver and kidney are increased as

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well. We also report that cathepsin D levels in some tissues are increased following leupeptin administration.

MATERIALS AND METHODS

Leupeptin has obtained courtesy of Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo. It was injected from a stock of 20 mg/ml of saline.

DBA/2N male mice were obtained age-matched from Harlon/Sprague Dawley, Indianapolis, Indiana.

Tritiated hemoglobin substrate for cathepsin D (Hb hydrolase) assay has been prepared in our laboratory by the procedure of Hatcher et al (4) and assayed by the modification of Roffman and Greenbaum (5).

CBZ-Arg-Arg- naphthylamide was obtained from Bachem, Inc., (Torrence, CA) and used as substrate for Cathepsin B assay by the method of McDonald et al (6) and Barrett (7).

CBZ-Phe-Ala-CHN_2 was generously provided by Dr. Elliott Shaw, Brookhaven National Laboratory, Upton, N.Y. It was injected from a stock of 6 mg/ml DMSO.

Immediately following sacrifice by cervical dislocation, tissues were removed from the mice for enzyme assay. They were weighed, and homogenized in a measured amount of iced 0.1% Triton X-100 solution (usually 10 ml/gm of tissue) with a Brinkman Polytron Homogenizer set at 6 for 25 seconds. The homogenate was centrifuged in the cold and the supernatant collected and frozen for assay. Protein was estimated by the method of Lowry (8).

RESULTS

In order to define the "kinetics" of inhibition of cathepsin B *in vivo*, experiments were performed on age-matched DBA/2 mice that were sacrificed 2, 4, and 6 hours after the i.p. injection of 200 mg/kg of leupeptin. In addition, a second group of mice were injected i.p. with 60 mg/kg with CBZ-Phe-Ala-CHN_2, in 100% DMSO, and sacrificed on the same schedule. Mice were also injected with 100% DMSO in appropriate amount to serve as controls for the Z... injected animals. Heart, liver and kidney from these animals were assayed for cathepsin B.

The results seen in Table 1 demonstrate that the Z... compound is a good inhibitor of tissue cathepsin B and inhibits all tissues for at least 6 hours after injection. On the other hand cathepsin B activity in the liver and kidney was depressed for only 4 hours by leupeptin and appeared to have returned to control values by 6 hours. In the heart, however, no decrease in cathepsin B was noted and in fact there was an apparent increase in cathepsin

Table 1

Comparison of In Vivo Inhibition of Tissue Cathepsin B By Leupeptin and Z-Phe-Ala-CHN_2

TISSUE	TIME	LEUPEPTIN Sp. Act.	%	Z-Phe-Ala-CHN_2 Sp. Act.	%
Liver	Control	43 ± 1.3	--	31 ± 1.9	--
	2	19 ± 2.5	-56*	9.4 ± 2.9	-69*
	4	34 ± 0.7	-19*	11 ± 0.4	-65*
	6	45 ± 1.5	+6	13 ± 0.3	-57*
Kidney	Control	38 ± 2.9	--	42 ± 4.5	--
	2	19 ± 5.0	-50*	7.3 ± 1.9	-83*
	4	28 ± 8.3	-28	9.9 ± 2.1	-76*
	6	45 ± 2.7	+18	17 ± 3.0	-56*
Heart	Control	9.6 ± 2.0	--	8.3 ± 0.6	--
	2	12 ± 2.0	+24	3.1 ± 0.2	-64*
	4	13 ± 1.3	+40	3.5 ± 0.2	-59*
	6	15 ± 2.5	+56*	3.7 ± 0.1	-55*

Following the i.p. injection of 200 mg/kg leupeptin in saline or 60 mg/kg of Z-Phe-Ala-CHN_2 in 100% DMSO, 3 mice were sacrificed at each of the time periods (hours). Specific activity of cathepsin B is in m units/mg of protein (or nmol/min/mg protein) ± S.E.M. The percent change (%) represents the difference between the specific activity of the tissues from the drug-injected and the vehicle-injected animals (control). *$P < .05$.

B activity starting at 2 hours, which was statistically significant in the 6 hour samples.

In order to examine this apparent paradox further, the following experiment was performed; a group of DBA/2 mice were injected i.p. with 200 mg/kg leupeptin and sacrificed at 0.5, 1, 2, 4, 6 and 24 hours after injection. Heart, liver and skeletal muscle were assayed for cathepsin B. Muscle was studied because of the reports of cathepsin B's involvement in muscle wasting (9-12). Table 2 demonstrates quite clearly that cathepsin B is significantly inhibited in all three tissues for the first hour following injection. However, by the second hour, the levels in the heart have begun to return to control levels and by the 4th hour, the heart and muscle cathepsin B activity has returned approximately to control levels.

All tissues also showed an increase in Hb hydrolase activity compared to the controls, with the increase in liver being statistically significant at the 0.001 level (Table 3).

With 20 mg/kg as the dose of leupeptin instead of 200 mg/kg the decrease in cathepsin B activity in heart, liver and kidney lasted about as long as in

Table 2

Effect of 200 mg/kg Leupeptin on Cathepsin B Activity

TIME	HEART Sp. Act.	%	MUSCLE Sp. Act.	%	LIVER Sp. Act.	%
Control	8.9±0.8	--	5.0±03	--	47±2.3	--
0.5 hrs	3.9±0.4	-44**	1.6±0.1	-68***	8.5±1.2	-82***
1 hr	3.7±0.2	-42***	1.4±0.4	-70***	7.7±1.8	-84***
2 hrs	5.8±1.1	-35	2.1±0.5	-58**	10.2±4.4	-78***
4 hrs	7.8±0.6	-12	5.1±1.1	+2	19±5	-60**
6 hrs	12.5±0.5	+40*	7.5±0.3	+50*	46±3	-2
24 hrs	19.0±2.5	+108*	11.3±1.5	+130*	76±10	+61*

Following the i.p. injection of 200 mg/kg of leupeptin in saline, 3 mice were sacrificed at each of the time periods. Specific activity of cathepsin B is in m units/mg of protein (or nmol/min/mg protein) ± S.E.M. The percent change (%) represents the difference between the specific activity of tissues from leupeptin-injected and vehicle-injected mice (control). *P < .05, **P < .01, ***P < .005.

the same tissue at the higher dose (Table 4). Each of these three tissue exhibited a "rebound" to normal levels and above in about the same amount of time as previously when the high dose of pepstatin was used. However, the cathepsin B activity of striated muscle were depressed for a much longer period than with the 200 mg/kg dose level. In each of the tissues, the changes in cathepsin B activity were smaller with the 20 mg/kg dose level than with the 200 mg/kg dose level.

Table 3

Increase in Hemoglobin Hydrolase Activity Following Leupeptin Administration

	HEART	LIVER	MUSCLE
Control	690 ± 76	740 ± 40	160 ± 11
Tumor Bearer	856 ± 32	1315 ± 72	315 ± 90
% Change	24 ± 12	78 ± 11***	95 ± 45

The figures represent specific activity and percent increase ± S.E.M. hemoglobin hydrolase activity in tissues 24 hours after the injection of 200 mg/kg of leupeptin. Activity of hemoglobin hydrolase is in percent hydrolysis of substrate per mg of protein. ***P < .001.

Table 4

Effect of 20 mg/kg Leupeptin on Cathepsin B Activity

TIME	HEART Sp. Act.	%	LIVER Sp. Act.	%	KIDNEY Sp. Act.	%	MUSCLE Sp. Act.	%
Control	8.8±0.9	--	44±2.3	--	38±2.9	--	6.0±0.8	--
0.5 hrs	7.3±0.4	-16	17±0.4	-60***	16±3.0	-60**	4.7±0.7	-22
1 hr	9.4±0.4	+7	29±0.6	-34***	29±2.5	-24	3.5±1.6	-42
2 hrs	8.7±0.5	0	34±0.9	-22*	30±0.3	-21*	5.3±1.2	-12
4 hrs	10.4±0.7	+18	37±0.3	-15*	44±2.6	+15	3.2±0.2	-47*
6 hrs	10.0±0.4	+15	40±2.0	-9	49±0.7	+28*	4.4±0.3	-27
24 hrs	12.0±1.6	+38	49±1.9	+11	48±9.0	+25	5.4±0.2	+10

Following the i.p. injection of 20 mg/kg of leupeptin in saline, 3 mice were sacrificed at each of the time periods. Specific activity of cathepsin B is in mUnits/mg of protein (or nmol/min/mg protein) ± S.E.M. The percent change (%) represents the difference between the specific activity of tissues from leupeptin-injected and vehicle-injected mice (control). *P <.05, **P < .01, ***P < .005.

DISCUSSION

Katunuma (13) reported inhibition by leupeptin of cathepsin B in rat liver *in vivo*. Tanaka and coworkers, demonstrated (14) that leupeptin administered to cultured hepatocytes could alter protease activity by causing induction of a hemoglobin hydrolase activity. The induction was blocked by inhibitors of protein synthesis and was rather specific in that there was no induction of acid phosphatase or cathepsin B. These investigators concluded that the induced enzyme had characteristics of both cathepsin L & D. Our data extend the *in vitro* findings of Tanaka et al. to the intact mouse. We have demonstrated that leupeptin causes an increase in cathepsin D-like activity (Hb-hydrolase) particularly in liver. Also, we show for the first time, that leupeptin paradoxically elevates cathepsin B activity in the heart, kidney, liver and striated muscle of the intact mouse. At dose levels of 200 mg/kg of leupeptin, the heart was the most sensitive of the tissues, elevation of activity occurring 2 hours following leupeptin administration. At 20 mg/kg dose of leupeptin, heart, liver and kidney again show elevated levels of cathepsin B. However, at this dose, striated muscle cathepsin B appears to be reduced in activity over the 6 hour period and returns to normal by 24 hours.

The question arises as to the mechanism by which leupeptin exerts its paradoxical effect. According to Tanaka et al (14), this is a true induction since in the cultured hepatocyte, cyclohexamide, an inhibitor of protein synthesis, prevents leupeptin-induced effect increase in hemoglobin hydrolase activity. Furuno et al (15) has shown in rats that lysosomal proteases move from their original organelles into autolysosomes following leupeptin administration. Thus, one possibility is that if a natural inhibitor of cathepsin B is present in the initial lysosomal site but not in the autolysosomes, increased protease activity might be noted. An additional possibility is that leupeptin stimulates the formation of a thiol activator which stimulates cathepsin B, although this seems unlikely in view of the increase in hemoglobin hydrolase, which is not a thiol activated enzyme.

Whatever the mechanism, the dramatic effect of cathepsin B stimulation must be taken into consideration when attempts at inhibiting cathepsin B or related thiol proteases in vivo or in cell culture are made using leupeptin. Our findings, together with those of Tanaka et al. and Furuno et al. (14, 15) clearly demonstrate that leupeptin or its metabolites have effects on the cell which are complex and not simply those of an antiprotease. Leupeptin is currently under study as an agent to alter dystrophy in muscles of chickens and mouse models (8-11), on presumption that it acts by inhibiting cathepsin B and related enzymes. Our current findings would suggest that in the mouse, leupeptin, depending on the concentration in the body or tissue, can actually elevate cathepsin B activity in several tissues including muscle. This action may limit the effectiveness of leupeptin as an anti-dystrophic agent as well as an antiprotease in vivo.

It is of interest that CBZ-Phe-Ala-CHN_2 when administered to mice is a good inhibitor of tissue cathepsin B. In contrast to leupeptin, no evidence of paradoxical enhancement of protease activity was noted. This agent should be considered with E-64 (7) as possibly having use as an anti-cathepsin B agent in diseased states.

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