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Hemoglobin-degrading Enzymes in Experimental Subcutaneous Hematomas*

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Summary. The activities of heme oxygenase and biliverdin reductase were determined in subcutaneous (s.c.) hematomas of rats after different periods of vital time. The postmortem stabilities of the heme-degrading enzymes were also studied by keeping the rats with vital hematomas for 1–5 days at temperatures of +4°C and +22°C. A tenfold increase of heme oxygenase activity over the starting level was observed in 2–9-day-old vital hematomas, when the specimens were taken immediately after death. Biliverdin reductase showed only negligible changes. Postmortally, heme oxygenase activity started to decrease in hematomas immediately at +22°C and from day 2 on at +4°C.

Key words: Hematoma – Heme oxygenase activity – Biliverdin reductase

Zusammenfassung. Die Aktivitäten von Hämi-Oxygenase und Biliverdin-Reductase wurden in subcutanen (s.c.) Hämatomen bei Ratten nach verschiedenen Vitalzeitperioden bestimmt. Die postmortale Stabilität der hämi-degradierenden Enzyme wurde ebenfalls untersucht, indem die Ratten mit vitalen Hämatomen 1–5 Tage bei +4°C bzw. +22°C gehalten wurden. Eine zehnfache Steigerung der Hämi-Oxygenaseaktivität über das Ausgangsniveau hinaus wurde in Hämatomen von 2–9 Tagen Alter wahrgenommen, wenn die Proben unmittelbar nach dem Tod entnommen waren. Biliverdin-Reductase wies nur geringfügige Veränderungen auf. Postmortal begann die Hämi-Oxygenaseaktivität in den Hämatomen bei +22°C unmittelbar bzw. bei +4°C vom zweiten Tag an herabzugehen.

Schlüsselwörter: Hämatome, Vitalzeitperioden – Hämioxygenaseaktivität – Biliverdin-Reductase

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The estimation of the age and vitality of different lesions has always been a problem in forensic medicine. For the timing of the wounds several histochemical enzyme methods have been presented [6, 7]. They, however, have not been easy to apply for hemorrhages or hematomas, in which the zone between living and dead tissues is not very clear-cut, and large amounts of hemoglobin may be present. In hematomas, reactions resulting in degradation and removing of extravasated hemoglobin start soon after the injury.

Heme oxygenase and biliverdin reductase form a major system for the degradation of hemoglobin and its derivatives to bile pigments in vivo [8, 9]. Heme oxygenase, being the rate-limiting enzyme, catalyzes the oxidation of heme at the α-methine bridge to form equimolar amounts of biliverdin and carbon monoxide. The biliverdin is subsequently reduced to bilirubin by the soluble biliverdin reductase.

While heme oxygenase activity appears to be present physiologically in high concentrations in the spleen, liver, and bone marrow, biliverdin reductase is more widely distributed and is present in great abundance in most tissues in relation to microsomal heme oxygenase [10]. The heme oxygenase activity is primarily controlled by the amount of heme that is offered for catabolism.

In s.c. hematomas, the conversion of heme to biliverdin and bilirubin is catalyzed enzymatically by heme oxygenase and biliverdin reductase brought to the site by macrophages [5].

In the present work it was studied whether the changes in heme-degrading enzyme activities might be useful for the timing of hemorrhages.

Material and Methods

The hematomas were induced by dissection of a s.c. artery in the pelvic limb of rats with a injection needle. Usually round s.c. hematomas with a diameter of about 2 cm were obtained.

The hematomas were stored at −70°C or in liquid nitrogen. Before analysis they were thawed at room temperature. After weighing the hematomas were scissored in 2 vols. of 0.9% NaCl into small pieces and homogenized with Potter-Elvehjem homogenizer. Heme oxygenase and biliverdin reductase activities were determined on 20,000 g supernatant fraction of the homogenates. For the measurement of heme oxygenase and biliverdin reductase activities, a Gilford model 2000 spectrophotometer, equipped with a constant temperature cuvette chamber, was used. Formation of bilirubin was determined from the increase in optical density at 468 nm, at which wavelength the pigment absorbed maximally in the incubation mixture used [8]. Enzyme activities were calculated from the maximal reaction rates.

The enzyme activities were expressed as pmoles bilirubin formed per 1 mg protein in 1 min. The protein concentration was assayed according to Lowry et al. [3].

Statistical analysis was performed using Student's *t*-test.

Results

Specimens were taken immediately after the vital period and stored at −70°C. In 15-min-old hematomas the heme oxygenase activity was rather low, 1.25 ± 2.0 pmol bilirubin/mg protein/min. Heme oxygenase activity increased rapidly during day 2 up to tenfold as compared with the 15 min level (Table 1). Thereafter, the activity remained at the same level, until on day 9 an additional

Table 1. Heme oxygenase and biliverdin reductase activities pmol bilirubin/mg prot./min in vital s.c. hematomas of age from 15 min to 21 days. Means, standard deviations (SD), and number of experiments are given. Specimens are taken immediately after death and stored at −70° C

Enzyme	Age of hematomas								
	15 min	1 day	2 days	3 days	5 days	7 days	9 days	14 days	21 days
Heme oxygenase	1.25 ±2.02	3.19 ±1.32	15.5 ±8.3*	14.5 ±7.2*	16.0 ±6.2*	17.3 ±6.9*	21.7 ±5.7*	8.2 ±3.2	2.1 ±2.2
Biliverdin reductase	30.1 ±9.9 ($n = 6$)	20.1 ±12.1 ($n = 13$)	23.2 ±8.4 ($n = 6$)	33.4 ±9.9 ($n = 8$)	37.9 ±10.0 ($n = 6$)	32.6 ±13.0 ($n = 9$)	75.9 ±22.5 ($n = 9$)	48.5 ±21.4 ($n = 11$)	49.1 ±12.2 ($n = 6$)

* $P < 0.001$

Table 2. Heme oxygenase and biliverdin reductase activities in 7-day-old vital s.c. hematomas of rats from which the specimens were taken after 1–5 days of postmortem storage at temperatures of +4°C or +22°C. The activities are expressed as percentages of the activities of specimens which were take immediately after death and stored at −70°C ($n = 3$)

Enzyme	Postmortem storage			
	Temperature °C	1 day %	3 days %	5 days %
Heme oxygenase	+4	101.2	51.3	5.4
	+22	10.6	0	0
Biliverdin reductase	+4	252.5	176.7	114.7
	+22	152.5	162.6	100.0

increase up to 15 times of the starting level was observed. From then on heme oxygenase activity began to decrease and was at the starting level in 21-day-old specimens.

Biliverdin reductase activity was 30.1 ± 9.9 pmol bilirubin/mg protein/min in 15-min-old hematomas and remained roughly at the same level during week 1. In 9-day-old hematomas the activity increased up to twofold as compared with the starting level. After that, the biliverdin reductase activity returned to the values observed in younger hematomas.

The postmortem stabilities of heme oxygenase and biliverdin reductase activities at different temperatures are described in Table 2. Rats with s.c. hematomas of 7 days duration were stored postmortally at +4°C or +22°C temperatures for 1–5 days. Heme oxygenase activity decreased to 11% during day 1 at +22°C and to 5% with in 5 days at +4°C. In biliverdin reductase activity, a rather slow decrease was observed after a transient rise.

Discussion

As known since Virchow, bilirubin crystals are formed in local blood extravasations [11]. The degradation of hemoglobin heme to bilirubin is physiologically catalyzed by the heme oxygenase-biliverdin reductase system, which is most active in spleen, liver, bone marrow, and other tissues involved in the degradation of heme compounds in vivo [8–10]. While this enzyme activity is very low in native macrophages, it may be stimulated by exposure of these cells to heme pigments in vivo [5]. The nature of this stimulation seems to be a substrate-mediated enzyme induction. Therefore, the time (2 days) necessary for the increase of heme oxygenase seems to be dependent on the time needed to mobilize phagocytic cells to the hematoma site as well as on the time needed for enzyme induction in these phagocytic cells. In fact, phagocytes are known to appear in hemorrhagic areas 1 day after injury [4]. After induction of the appropriate lysosomal [1] and microsomal enzymes, the hemoglobin is degraded into its catabolites, bilirubin, iron, and amino acids, which are then gradually re-

leased into the extracellular space. The successive enzymatic conversion by phagocytes of the ingested hemoglobin (dark red) methemoglobin or hematin (dark brown) to biliverdin (green-blue) and eventually to bilirubin (yellow) is responsible for the characteristic color play in s.c. bruises. Hemosiderin-laden macrophages (brown) may contribute to the color spectrum [4]. Furthermore, the activity of heme oxygenase starts decreasing at the time when most of hemoglobin of these experimental hematomas has disappeared [2].

The changes of heme oxygenase activity are more marked and more clearly time-connected than changes in biliverdin reductase activity. This is in accordance with the earlier reports [10]. Heme oxygenase is the rate-limiting enzyme of the enzymatic heme degrading pathway [8]. On the contrary, biliverdin reductase is present in excess in a number of mammalian tissues [9].

The postmortal storage experiments at temperatures of +4°C and +22°C gave results in accordance with the earlier observations about the stability of these enzymes in vitro [8, 9]. In case there is a possibility to take samples soon after death the heme oxygenase activity could be used according to these experimental data to differentiate 1-day-old or younger hematomas from those 2–9 days old. Also in s.c. hematomas, in which the morphological changes clearly show the age older than 1 day (e.g., plenty of Berlin blue-positive macrophages, connective tissue formation, new capillaries, etc.) low heme oxygenase activity might indicate that such hematomas are more than 2 or 3 weeks old. However, the time-dependent changes of enzyme activities observed in experimental hematomas of rats are not necessarily equivalent to human hematomas. Furthermore, the localization and the size of hematomas might be of importance. In this study, the localization and size of hematomas were identical. It could be expected that the time sequence of changes might be different depending on the size of hematomas. In any case, the usefulness of heme oxygenase measurements in the determination of the age of hematomas postmortally will be limited to 1 day. Furthermore, the storage temperature of the bodies will be of importance in the evaluation of enzymatic findings.

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