Table 4. LDL-induced increase (↑) and decrease (↓) of cellular metabolites in various tissues. PIP_2: phosphatidylinositol-diphosphate; DAG: diacylglycerol; IP_3: inositoltriphosphate; $(Ca^{2+})_i$: intracellular free calcium.* Preliminary results.

Metabolite	Platelets	Vascular muscles	Fibroblasts	Brain slices*
PIP_2	↓	↓	↓	
DAG	↑	↑	↑	
IP_3	↑	↑	↑	↑
$[Ca^{2+}]_i$	↑	↑	↑	

L. Block and A. Pletscher (not printed). In contrast to high density lipoprotein (HDL_3), human low density lipoprotein (LDL), which binds to specific sites of human platelets (K_D $3-6 \times 10^{-8}$ M), induced a shape change reaction, measured by densitometry and verified by electron microscopy. This effect was accompanied by an increased generation of diacylglyceride, phosphatidic acid and inositol 1,4,5-trisphosphate (IP_3), by a decrease in phosphatidylinositol 4,5-bisphosphate (PIP_2), and by an elevation of cytosolic free calcium concentration, $(Ca^{2+})_i$. The rise of $(Ca^{2+})_i$ was more pronounced in calcium-containing media, but was still significant in the absence of extracellular calcium, suggesting that LDL mobilizes calcium from intracellular stores. Both the functional and biochemical changes occurred at the same order of magnitude (1–40 µg/ml) of LDL and with similar time courses, and both were counteracted by HDL_3 (20–100 µg/ml), which is known to act as an inhibitor of LDL binding. The LDL-induced changes were paralleled by those of thrombin except that HDL_3 caused no inhibition in the case of thrombin. Inhibition of LDL-induced activation was also seen in the presence of albumin. The inhibitory action of plasma proteins may explain the lack of responsiveness of platelets to LDL when tested in platelet rich plasma.

These results indicate that LDL causes rapid platelet activation by stimulation of phospholipase C, resulting in an enhancement of phosphatidylinositol (PtdIns)-turnover with a rise in $(Ca^{2+})_i$. Further studies showed that low concentrations of LDL also activate the (PtdIns)-cycle in rat arterial smooth muscle cells, human skin fibroblasts, vascular endothelial cells, and, according to preliminary experiments, also in slices of rat brain (table 4) [1].

Therefore, low concentrations of LDL seem to bring about cell activation, possibly in a hormone-like way. This effect could play a role in pathophysiological processes (e.g. in arteriosclerosis) and here again, platelets might serve as a useful model.

'Praise' of platelets as models

The rationale for using platelets as models for cells can be summarized as follows:

1) Platelets are easily obtainable in native form from humans and, in contrast to animal cells, are undoubtedly representative for man.
2) It can be assumed that certain pathological disturbances of neurotransmitter dynamics and receptor function in mental and cardiovascular disorders are of a generalized nature. Platelets can therefore play a role in elucidating pathophysiological mechanisms and may serve as diagnostic tools or in some cases as monitors of therapy.
3) Platelets may be used for pharmacological screening (e.g. for 5 HT uptake inhibitors, amine releasers, receptor-active compounds), thus reducing the number of animal experiments required.

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Current activities in the Department of Research of the University Hospital, Basel

Human growth hormone in urine: development of an ultrasensitive radiometric assay

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Summary. An immunoradiometric assay for human growth hormone (HGH) has been developed which has a detection limit of 1 ng/l and can measure HGH in unextracted urine from normal children and adults. The assay is based on a two-step procedure, using a solid-phase goat-anti-HGH immunosorbent for immunoextraction and [^{125}I]-labeled monoclonal HGH-antibody for detection and quantification. The assay is not affected by urea, NaCl or changes of pH from 5–8. The mean urine HGH concentration in normal children is 6.78 ± 7.6 (SD) pg/ml, in patients with HGH-deficiency 1.3 ± 0.9 pg/ml which increases to 11.7 ± 13.4 pg/ml on the day of growth hormone injection.
Key words. Human growth hormone (HGH); urine; IRMA; ultrasensitive assay.

Human growth hormone (HGH) is under metabolic and hormonal feedback control. At the pituitary level, the regulation appears to be mainly mediated through the interactions of somatostatin and growth hormone-releasing hormone (GHRH)[14, 16]. The secretion is episodic and modulated by a sleep-wake-rhythm. Multiple feedback signals via blood glucose, amino acids, somatomedin and HGH itself render the control of secretion very complex. The sequence analysis and chemical synthesis of GHRH as well as the availability of biosynthetic HGH have led to a reinvestigation of several aspects of the physiology and pathophysiology of HGH secretion. A number of studies have shown that the diagnosis of HGH-dysfunction, based on pharmacological (e.g. insulin-tolerance, arginine infusion or clonidin) or physiological (e.g. 24-h profiles) tests is unreliable[3, 18]. This applies not only to the broad range between normal HGH secretion and complete HGH deficiency but also to the follow-up of acromegalic patients during treatment[17]. Furthermore, HGH is considered to be an important counterregulatory hormone in diabetes mellitus[11].

Currently available assays for HGH usually have a detection limit of 0.5–1.0 µg/l plasma although more sensitive assays using monoclonal antibodies have been described[9]. Circulating HGH levels are below the limits of detection of conventional RIAs for most parts of the circadian cycle[5, 15]. This lack of sensitivity of HGH assays has rendered their application to urinary samples impractical. However, it has been demonstrated that the hormone is excreted in urine, and it can be detected if present in large amounts, but the absolute levels reported are conflicting[1, 2, 4, 8, 10].

It was the purpose of this study to develop a sensitive assay for HGH applicable to both plasma and urine[6]. The principle of the assay is based on the methodology of the radiometric assay using labeled antibodies[12, 19].

Materials and methods. Peptides. HGH was a commercially available pituitary extract (Nanormon, Nordisk). Labeled HGH was prepared by the conventional chloramine T-method.

Antibodies. Monoclonal HGH-antibodies were obtained from Medix Biochemica, clone code 5801. Polyclonal HGH-antibodies were raised in minigoats by multiple-site sc injection of 200 µg (1st injection), 100 µg (2nd–4th injection) and 50 µg (from 5th injection) of HGH dissolved in saline and mixed 1:1 with complete (1st and 2nd injection) or incomplete (from 3rd injection) Freund's adjuvant. The injections were carried out every 6–8 weeks and the animals were bled 2–4 weeks later. The resulting antiserum of the 5th bleeding bound 50% of approximately 200 pg (7000 cpm) [^{125}I]-labeled HGH at a final dilution of 1:500,000.

Iodination of monoclonal anti-HGH antibody. The iodination was carried out using the conventional chloramine T method: 1 µCi of Na^{125}I and 40 µg of anti-HGH antibody in 40 µl 0.3 M Na-phosphate buffer, pH 7.3, were exposed to 20 µg of chloramine T in 10 µl of the same buffer, by vortexing for 25 s. The reaction was stopped by adding 25 µl of a saturated tyrosine solution. After a further 25 s, 250 µl of PBS (0.05 M Na-phosphate buffer, pH 7.5, plus 0.9% NaCl) containing 0.2% BSA were added, and the iodinated mixture was applied to a Sephadex G-15 column (8.5 × 1 cm) primed with PBS containing 1% BSA. The labeled antibody was eluted with PBS.

Preparation of anti-HGH immunosorbent. Non-sedimenting polyacrylamide beads (6 ml containing ~ 0.2 g; EIR, Würenlingen) were washed twice with 0.03 M Na-phosphate buffer, pH 6.3 and resuspended in 6 ml of the same buffer. Polyclonal HGH-antiserum (0.1 ml) was added to the suspension, followed by 2 × 10 mg 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDAC; Sigma). The suspension was kept at 4 °C overnight, centrifuged and washed with 12 ml of 0.05 M Na-phosphate buffer, pH 7.5, containing 0.15 M NaCl, followed by 12 ml 0.25 M Na-phosphate buffer, pH 7.5, containing 1 M NaCl. The pellet was resuspended and kept overnight at 4 °C in 12 ml 0.05 M Na-phosphate buffer, pH 7.5. Before use, the suspension was centrifuged and the pellet resuspended in assay diluent (0.05 M Na-phosphate, pH 7.5, containing 0.9% NaCl, 0.1% BSA, 0.01% Na-ethylmercurithiosalicylate and 0.1% Triton X-100).

Titration of immunosorbent. Serial dilutions of immunosorbent were prepared in assay diluent; 50 µl of each dilution was incubated in 1 ml diluent containing approximately 5000 cpm of [^{125}I]-HGH, with or without 250 pg of unlabeled HGH. The dilution of immunosorbent binding more than 80% of the label in the presence of unlabeled HGH was chosen for the assay. This allows a working range for the assay from 0 to 250 pg HGH per tube.

HGH assay. For urine, a total incubation volume of 5 ml containing urine diluted 1:1 with diluent was used (in some experiments, this volume was doubled). HGH standard (2–250 pg) and urine samples were incubated with 50 µl of the pretitrated immunosorbent. The tubes were shaken at 4 °C for 15–18 h, centrifuged at 2000 rpm for 20 min at 4 °C and washed with diluent. The pellets were resuspended in a final volume of 1 ml assay buffer containing approximately 10,000 cpm of monoclonal [^{125}I]-HGH-antibody. After shaking for a further 12–18 h at 4 °C, the tubes were centrifuged, and the pellets washed twice with 1 ml of diluent and counted in a γ-counter.

Urine samples. After determination of the volumes, first morning voiding urine and 24-h urine (stored at 4 °C) were kept frozen in 20-ml aliquots without preservatives until assay. HGH-free urine was prepared by incubation with excess immunosorbent.

Results. Standard curves. Figure 1 shows the linear correlation between HGH content and the [^{125}I]-labeled antibody bound to the immunosorbent over the range from 2 to 250 pg HGH per tube. The interassay coefficient of variation was 14.1% at a mean concentration of 26 pg HGH/2.5 ml of urine. The intraassay coefficient of variation was below 3.5% for HGH concentrations of 31, 250 and 1000 pg/5 ml.

Sensitivity. The minimal detectable amount of HGH was studied by addition of 0.8 pg/ml or 1.6 pg/ml of HGH to hormone-free urine or diluent. Comparison of the increase in cpm over basal values + 3 SD (which is defined as sensitivity of the assay) shows that there is no difference between urine and diluent (fig. 2). By extraction of 5–10 ml of urine, as little as 0.5 pg (2 × 10^{-17} mol) HGH per ml urine can therefore be detected.

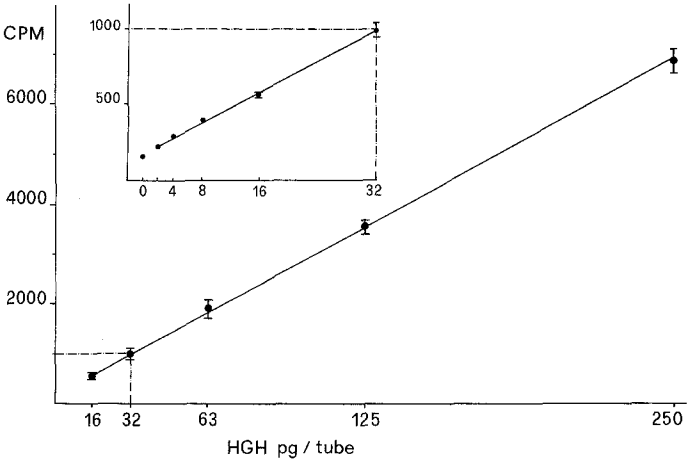

Figure 1. Linear correlation between radioactivity (cpm) bound to the immunosorbent and HGH content per tube (mean ± SD).

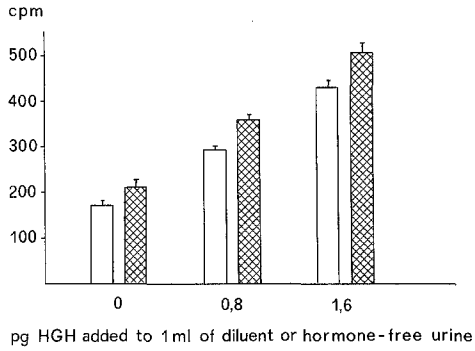

Figure 2. Sensitivity of the radiometric assay, using an incubation volume of 5 ml, containing 2.5 ml of assay buffer (white) or 2.5 ml of hormone-free urine (hatched).

Effect of urea, NaCl and pH of urine samples on [^{125}I]-HGH-antibody binding in the radiometric HGH assay. Urine samples were either free of HGH or contained 31, 125 or 250 pg HGH added.
[a] Mean ± SD.

Treatment		[^{125}I]-HGH-antibody bound (cpm)[a]		
		HGH free	HGH added	
			250 pg HGH	
Urea	0	486 ± 38	4485 ± 45	
	0.2 M	447 ± 3	4483 ± 32	
	0.4 M	469 ± 9	4271 ± 53	
			31 pg HGH	125 pg HGH
NaCl	100 mosmol	53 ± 3	1056 ± 24	4163 ± 22
	500 mosmol	73 ± 12	1209 ± 12	4715 ± 34
	750 mosmol	46 ± 4	1228 ± 10	4662 ± 31
	1000 mosmol	37 ± 8	1183 ± 17	4531 ± 33
pH	5	52 ± 6	1127 ± 6	4366 ± 18
	6	48 ± 4	1189 ± 11	4513 ± 19
	7	43 ± 6	1184 ± 16	4634 ± 27
	8	47 ± 4	1232 ± 4	4689 ± 21

Non-specific interferences. The assay is not affected by urea (up to 0.4 M), NaCl (up to 1000 mosmol) or pH (between 5 and 8), as shown in the table.

Application of the assay to urine. For practical reasons, a total incubation volume of 5 ml containing 2.5 ml of urine was used for immunoextraction. A mean concentration of 6.78 ± 7.6 pg/ml HGH was found in 98 24-h or first morning voiding urine samples from children of different age groups without an endocrinopathy. The mean concentration of 13 samples from children with an HGH-deficiency was 1.3 ± 0.9 pg/ml on the day without treatment, and 11.7 ± 13.4 pg/ml on the day of treatment. The mean HGH excretion over 24 h in children without endocrinopathy was 6.6 ± 21 ng, and in 3 acromegalics 73, 114, and 208 ng respectively. In children with HGH-deficiency, the 24-h urine value on the day without therapy was 1 ± 0.7 ng HGH and on the day of therapy 6.9 ± 8.2 ng. There was no correlation between urinary HGH and creatinine concentration, osmolarity and pH of urine. In order to avoid under- or overestimation of HGH, the hormone was determined 1) in untreated urine; 2) after addition of 20 pg of HGH to untreated urine; 3) after removal of endogenous hormone by extraction with excess immunosorbent; 4) after addition of 20 pg of HGH to extracted urine.

Discussion. Previous attempts to measure HGH in urine were hampered by the lack of sensitivity of the methods used. Interference by urea, osmolarity and pH in untreated urine required extraction procedures which are difficult to control. In untreated urine, only acromegalic samples contain enough immunoreactive HGH to the detected in a direct assay[1, 2, 10]. Baumann and Abramson[2] found that the monomeric form of HGH (20 and 22 K) are similarly represented in urine as in plasma. By extracting large urine volumes, the authors calculated that approximately 0.01% of the HGH produced daily is finally excreted in urine[2]. Whether urinary HGH-secretion reflects a total daily HGH-production and can be used for diagnostic purposes remains to be shown. In analogy to the assay of urinary gonadotrophins and steroids (i.e. free cortisol), and from the preliminary results in normal children compared to those with pituitary insufficiency and acromegalics, analysis of HGH excreted in urine appears to be a useful addition to plasma assays. It is obvious that HGH in urine can only reflect a total production and does not give any clue to episodic secretion and immediate metabolic controls. However, experience of HGH-treatment in HGH-deficient patients has shown that a daily dose or injections 3 times a week of HGH lead to catch-up growth and to a normalization of growth rate in affected children. It appears therefore that at least for the growth effect of HGH, the total amount of hormone available rather than the pulsatility of its secretion is of importance. Urinary HGH-excretion could be a useful tool for monitoring GHRH-therapy. It also has a potential application for confirming the diagnosis and for the follow-up of acromegalic patients. The determination of the total daily HGH production in diabetes mellitus would be valuable to evaluate its pathophysiological significance for the control of the disease.

Preliminary results[6] suggest that this radiometric assay can be applied to plasma, resulting in a considerable improvement in sensitivity. This would then reduce plasma volumes required for multiple (profile) assays and might help to elucidate some aspects of the pathophysiology of HGH-secretion.

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