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Degradation products of poly(ester-ether-ester) block copolymers do not alter endothelial metabolism *in vitro*

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In this paper we report experimental results from specific biocompatibility tests on two different classes of tri-block poly(ester-ether-ester) copolymers, namely copoly(ε-caprolactone-oxyethylene-ε-caprolactone) and copoly(L-lactide-oxyethylene-L-lactide). In particular, the human umbilical vein endothelial cells (HUVEC) metabolism has been studied in the presence both of the copolymers and of their hydrolytic breakdown products. The release of prostacyclin (PGI_2) and angiotensin II (AII) has been measured at different times for copolymers with different composition and hydrophilicity. The results obtained seem to indicate that the block copolymers of both series do not alter the PGI_2 and AII release by HUVEC populations *in vitro*. Similar results are found also after a prolonged contact between the cells and the hydrolytic breakdown products of the copolymers. Furthermore, some of the copolymers studied significantly stimulate the release of either PGI_2 or AII. This finding might point towards the use of these copolymers as bioactive materials in the field of cardiovascular devices.

1. Introduction

It is well known that, among the different classes of biodegradable polymers, polyester homopolymers and polyester-based copolymers are the most widely studied ones [1]. Apart from favourable physicochemical and mechanical properties, the most important requirement for a biodegradable polymer is its biocompatibility in a specific environment, together with the nontoxicity of its degradation products.

The synthesis, the physicochemical characterization and the biocompatibility of two different classes of poly(ester-ether-ester) block copolymers, obtained by reaction of preformed poly(ethylene glycol) (PEG) with either ε-caprolactone (CL) or L-lactide (LA) monomers, have been previously reported [2, 3]. More recently the biodegradation characteristics of the same copolymers were evaluated *in vitro* in the presence of murine fibroblasts [4]. The two series of copolymers studied will be indicated as PCL-POE-PCL and PLA-POE-PLA in this paper.

To better define the biocompatibility characteristics of the copolymers, we report experimental data on two aspects of the endothelial cell metabolism, i.e. prostacyclin (PGI_2) [5] and angiotensin II (AII) [6] release, in the presence of both series of copolymers and of their hydrolytic breakdown products. Such products are 6-hydroxyhexanoic acid for PCL-POE-PCL copolymers and L-lactic acid for PLA-POE-PLA copolymers.

2. Materials and methods

2.1. Copolymer synthesis

The chemical synthesis of the PCL-POE-PCL and PLA-POE-PLA tri-block copolymers was carried out according to the general scheme shown in Fig. 1. The detailed experimental conditions of the synthesis have been already reported [2–4]. The copolymer degradation products, i.e. respectively 6-hydroxyhexanoic acid and L-lactic acid, are released according to the scheme shown in Fig. 2 [4, 7].

2.2. Biodegradation assay

The *in vitro* biodegradation assay of the copolymers is described elsewhere [4]. In short, human umbilical vein endothelial cells (HUVEC) were isolated from the umbilical cords immediately after delivery, following a standard protocol [8]. Culture medium (CM) was made of equal parts of M199 and RPMI 1640 media,

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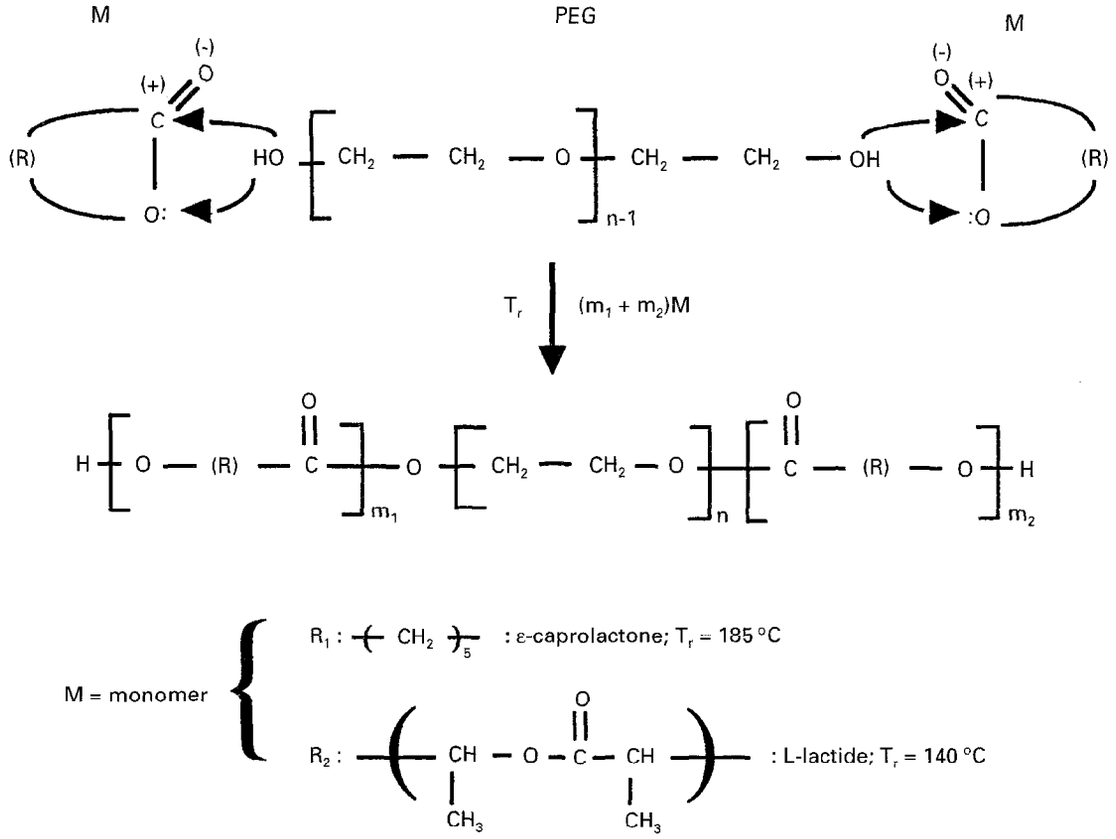


Figure 1 General scheme of reaction for the chemical synthesis of the PCL-POE-PCL and PLA-POE-PLA tri-block copolymers.

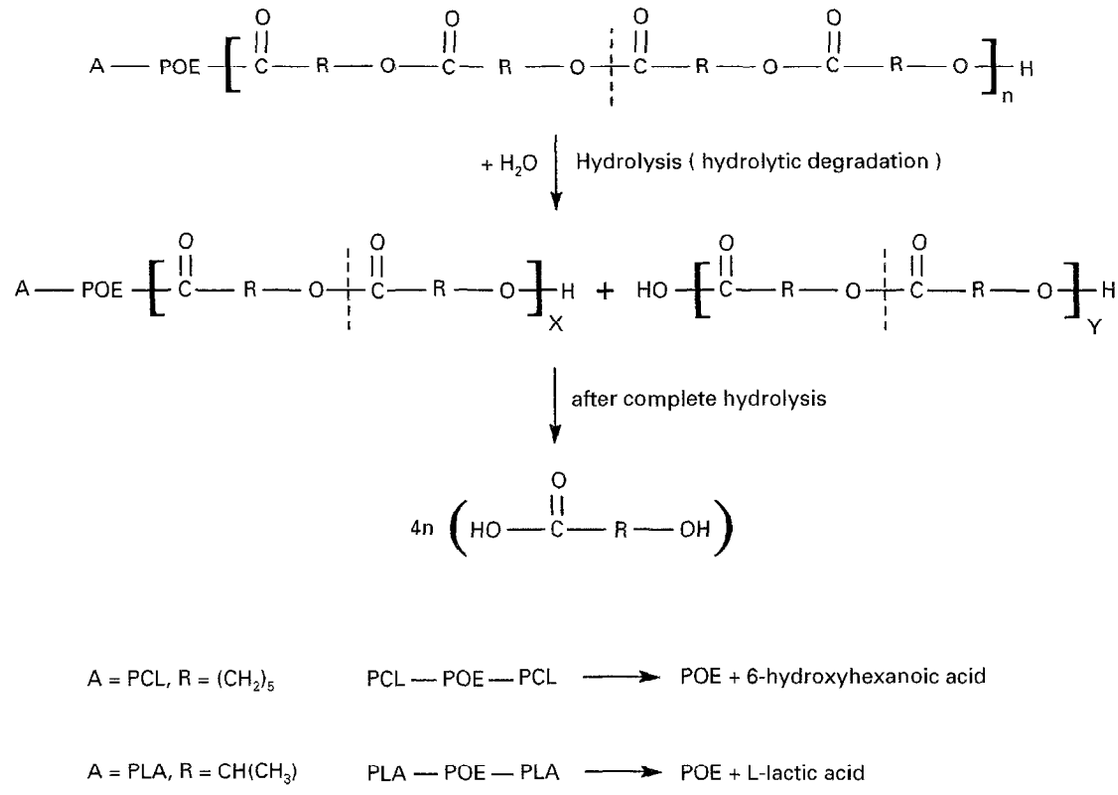


Figure 2 General scheme of the hydrolytic degradation of the PCL-POE-PCL and PLA-POE-PLA copolymers.

20 mM Hepes buffer, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 5 μg/ml fungizone and added with 20% pooled human serum.

HUVEC (seeding density = 5×10^3 cells per cm^2) were cultured on six-well culture plates for 4 days.

Pre-weighed copolymer samples (CL 24, CL 27, CL 28 [2] and LA 3, LA 4, LA 5 [3]) in the form of powder, sterilized by ethylene oxide, were then laid on to polycarbonate microporous (pore size 3 μm) inserts (Transwell, Costar, I) hanging from the top of the

culture wells. CM was added to completely cover the HUVEC and the materials. No material was added to the negative control (NC) wells.

At day 3, 5, 10 CM was completely withdrawn from the wells, replaced by fresh medium, and used both for HPLC analysis [4] and for PGI_2 and AII assays. At day 10 the HUVEC were trypsinized and counted by means of a haemocytometer. The PGI_2 and AII release per cell unit per well were calculated.

2.3. PGI_2 assay

PGI_2 release was measured, as previously described [9], by a Radio Immuno Assay (RIA) of 6-keto-$PGF_{1\alpha}$, the stable hydrolytic product of PGI_2 (Cayman Chemicals, Ann Arbor, USA).

2.4. AII assay

AII was assayed by a direct RIA for AII level determination in plasma (Angiotensin II Pasteur, E.R.I.A. Diagnostics Pasteur, France).

3. Results

3.1. PGI_2 release

At day 10 all the wells presented confluent HUVEC monolayers. The test wells of both copolymer series contained HUVEC populations that were statistically not different from the NC wells (t-test, $p > 0.05$, data not shown) [7].

PGI_2 release by HUVEC at days 3, 5, and 10 is shown in Fig. 3a, b. The most hydrophilic copolymers of both series, i.e. LA 4 and CL 24 [4], elicit a PGI_2 release that is constantly similar to that of their NC. The most hydrophobic copolymers, i.e. LA 3 and CL 27 [4], and those with an intermediate degree of hydrophilicity, i.e. LA 5 and CL 28 [4], seem to generally produce an increased PGI_2 release. At day 10, the general trend seems to be, for all the copolymers, towards PGI_2 values similar between the test and the NC wells. This is especially true for the PCL-POE-PCL series where no statistical differences between the samples are found at day 10 (Fig. 3b). The 2-factor Analysis of Variance (ANOVA, data not shown) on PGI_2 indicates that, in the case of the PCL-POE-PCL copolymers, neither the time nor the copolymer type factors influence PGI_2 release. When the PLA-POE-PLA copolymers are put in contact with HUVEC, only the type of copolymer factor has influence on PGI_2 (2-factor ANOVA, $p = 0.0001$). In Table I, PGI_2 release per cell unit at day 10 is given.

3.2. AII release

In the presence of the PLA-POE-PLA copolymers, the release of AII by HUVEC is characterized by a gradual slowing down of the values with time, until, at days 5 and 10, there is no significant difference between test and control wells (Fig. 4a).

The behaviour of the PCL-POE-PCL series seems to be quite similar to the previous one (Fig. 4b), with the exception of the copolymers CL 24 (at day 5) and CL 28 (at day 10). The 2-factor ANOVA on AII (data not shown) suggests that for the PLA-POE-PLA series both factors, time and type of copolymers, influence significantly AII release ($p < 0.001$). On the contrary, AII release by the PCL-POE-PCL series is significantly influenced only by the time factor ($p < 0.0001$). In Table I, AII release per cell unit at day 10 is reported.

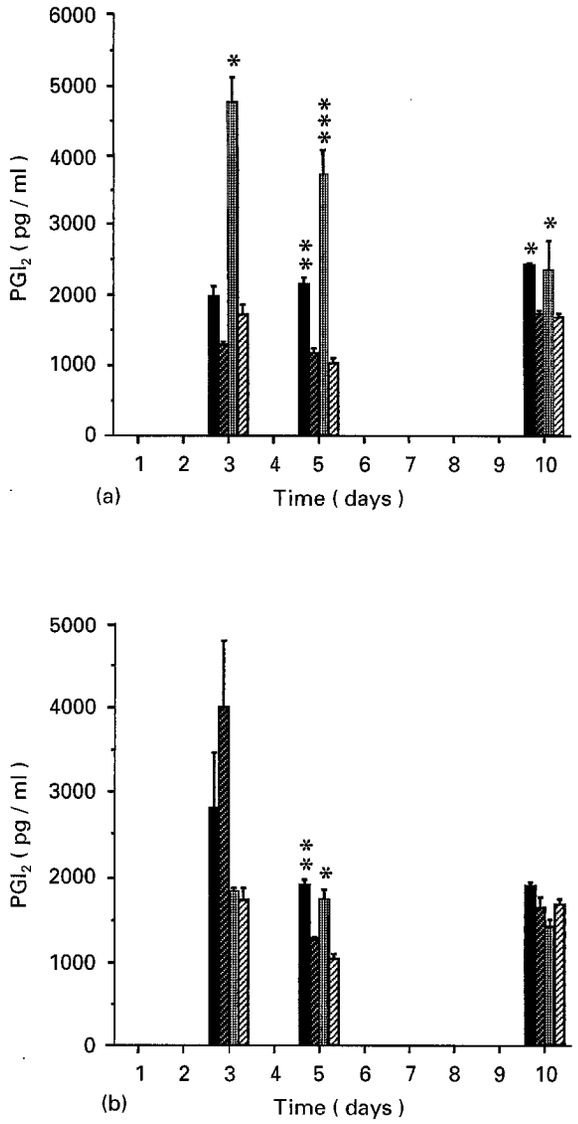
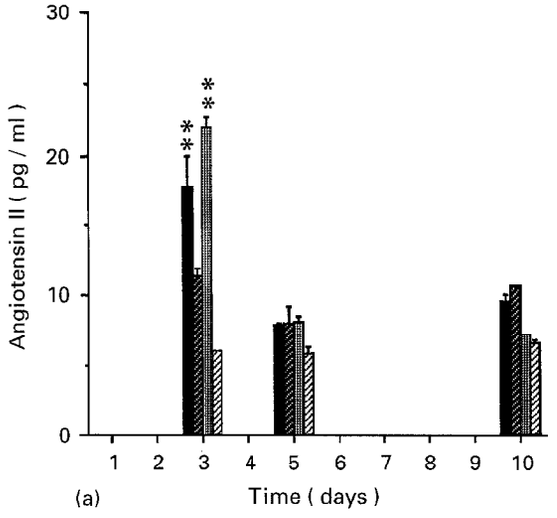


Figure 3 PGI_2 release by HUVEC in the presence of PLA-POE-PLA (a) and PCL-POE-PCL (b) copolymers. The data are the mean ± SE of four determinations. (■) LA 3, CL 27; (▩) LA 4, CL 24; (▦) LA 5, CL 28; (▨) NC (negative control). Fisher's PLSD test, significance level 5%, $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

TABLE I PGI_2 and AII release per endothelial cell unit (pg/ml per cell number) at day 10. The mean ± SE ($n = 4$) is expressed. Fisher's PLSD test, significance level 5%, $*p < 0.05$, $**p < 0.01$

	PGI_2	AII
LA 3	0.0196 ± 0.000014	0.78 ± 0.040
LA 4	*0.0143 ± 0.000028	0.88 ± 0.025
LA 5	0.0426 ± 0.0051	*1.31 ± 0.005
CL 27	*0.0185 ± 0.0003	0.79 ± 0.129
CL 24	**0.0130 ± 0.00072	0.39 ± 0.002
CL 28	*0.0225 ± 0.00097	*1.98 ± 0.086
NC	0.0334 ± 0.00061	0.65 ± 0.016



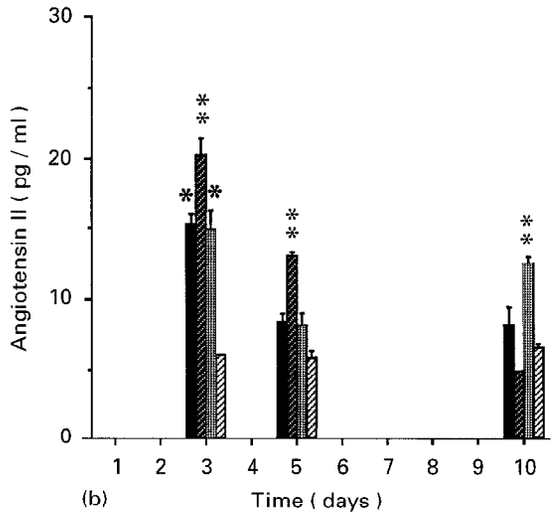
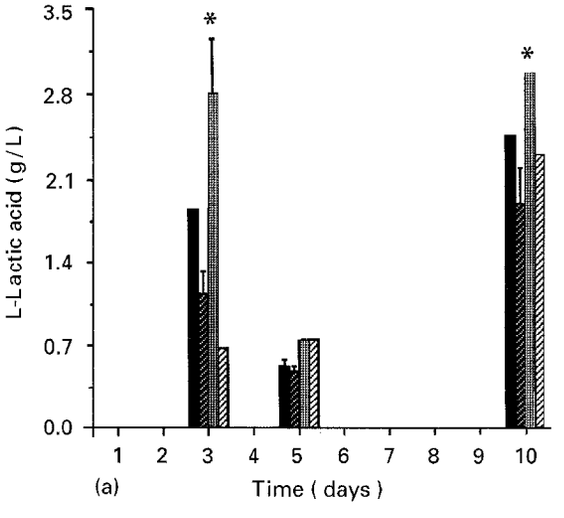


Figure 4 AII release by HUVEC in the presence of PLA-POE-PLA (a) and PCL-POE-PCL (b) copolymers. The data are the mean ± SE of four determinations. (■) LA 3, CL 27; (▩) La 4, CL 24; (▦) LA 5, CL 28; (▨) NC (negative control). Fisher's PLSD test, significance level 5%, $*p < 0.05$, $**p < 0.01$.



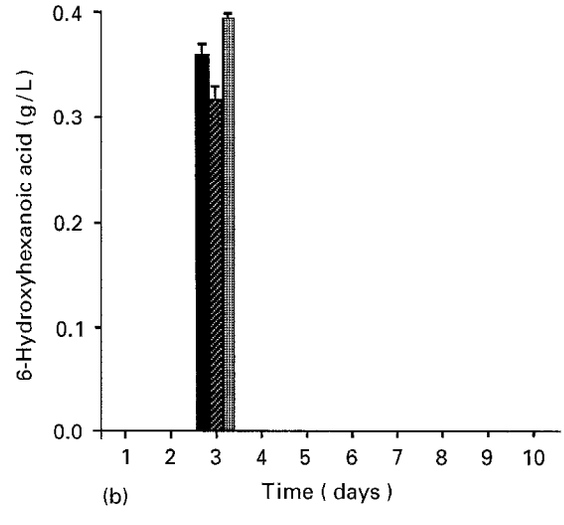


Figure 5 HPLC analysis of PLA-POE-PLA (a) and PCL-POE-PCL (b) degradation. The release of L-lactic acid (a) and of 6-hydroxyhexanoic acid (b) by the copolymers with time, in the culture medium and in the presence of HUVEC populations, is given. The data are the mean ± SE of four determinations. (■) LA 3, CL 27; (▩) LA 4, CL 24; (▦) LA 5, CL 28; (▨) NC (negative control). Fisher's PLSD test, significance level 5%, $*p < 0.05$, $**p < 0.01$.

3.3. HPLC analysis

The amounts (g/l) of L-lactic acid and 6-hydroxyhexanoic acid, the degradation products of PLA-POE-PLA and PCL-POE-PCL copolymers in the presence of confluent HUVEC populations, are shown in Fig. 5a, b. No 6-hydroxyhexanoic acid was detected at days 5 and 10 as well as in the control wells, where HUVEC without test material were present (Fig. 5b).

4. Discussion

Our results demonstrate that PGI_2 and AII release by HUVEC populations *in vitro* are not impaired by the presence of block copolymers of the series PLA-POE-PLA and PCL-POE-PCL (Figs 3 and 4). Endothelial cells are recognized as the active barrier between circulating blood and vessel wall. They are the site of an intense metabolic activity directed towards the prevention of thrombotic events. Endothelial cells can produce both vasodilators, such as PGI_2 [5] and endothelium-derived relaxing factor (EDRF), and vasoconstrictors, such as AII [6] and endothelin 1, that may interplay in the local control of the circulation. Furthermore AII and PGI_2 release are known to be mutually interrelated [10]. At days 3 and 5, after putting in contact the copolymers with the cells there are some significant differences in PGI_2 and AII release in the test wells if compared with the control wells. These differences seem to level off at day 10 in most cases, possibly indicating a metabolic adjustment of the cells to the copolymer degradation products. The copolymer hydrolytic breakdown products were detected and measured by HPLC analysis during the same time. They are lactic acid, CH_3–CH(OH)–COOH and 6-hydroxyhexanoic acid, HO–$(CH_2)_5$–COOH, respectively for the PLA-POE-PLA and the PCL-POE-PCL series. The time course of their release is shown in Fig. 5a, b. By comparing Figs 3a and 4a with Fig. 5a, it can be observed that the amounts of released PGI_2 and AII show a pattern

similar to that shown by the L-lactic acid detected in the wells. In particular, LA 4, the most hydrophilic copolymer of the series [4], releases by degradation less lactic acid than LA 3, the least hydrophilic one, and LA 5, the one with an intermediate hydrophilicity [4].

Accordingly, the breakdown products of LA 4 seem to stimulate PGI_2 release by HUVEC less than those of LA 3 and LA 5 (Fig. 3a). This effect is especially seen for AII release at day 3 (Fig. 4a). The stimulatory effect of LA 5 towards PGI_2 and AII release compared with LA 4 and LA 3 can be appreciated when the amounts of PGI_2 and AII per cell unit are reported (Table 1).

Similarly, if we consider in detail the influence of the PCL-POE-PCL copolymers on both PGI_2 and AII release, it can be noticed that both of them follow the trend CL 28 > CL 27 > CL 24 (Table I), which is similar to that found for the 6-hydroxyhexanoic acid release (Fig. 5b). This copolymer hydrolysis product was detected in the culture wells only at day 3, and not both in the control wells (only HUVEC) and in the test wells at days 5 and 10 (Fig. 5b). This finding seems to suggest an active metabolism of 6-hydroxyhexanoic acid by HUVEC. As far as we know, the cellular metabolic pathway of 6-hydroxyhexanoic acid has not yet been investigated and deserves further study.

There is an increasing interest towards the development of biodegradable materials for biomedical use. The poly(ester-ether-ester) block copolymers under study were already tested for cytotoxicity and cytocompatibility [2, 3] and their biodegradation behaviour was characterized both in the presence and in the absence of 3T3 fibroblast populations *in vitro* [4].

The specific biocompatibility testing presented in this paper is an attempt to closely simulate an *in vivo* situation under the hypothesis that our materials come in close contact with endothelial cells, e.g. in a cardiovascular device. If certain cellular responses, regarded as relevant and desirable for the cell type in contact with the medical device, are stimulated, the biomaterial can be regarded not only as biocompatible, but also as bioactive [11]. Biocompatibility testing involves mostly a process of exclusion of negative effects due to the biomaterials in cells and tissues. On the contrary, bioactivation involves an inclusion of positive stimulatory effects due to the biomaterials, and has to be considered as a part of a new idea of biocompatibility.

The results not only confirm the biocompatibility of the copolymers under study [2, 3], but also suggest that some of them can be regarded as bioactive materials. The cellular mechanisms, by which PGI_2 and AII release are stimulated by 6-hydroxyhexanoic acid and L-lactic acid, are still unknown and will be a matter of further investigation.

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